Novel aspects of nest defence in stingless bees

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Declaration

I declare that the work carried out in this thesis is entirely done by me, and that any help provided by other individuals with data collection and analysis is fully acknowledged.

I certify that this thesis has not and will not be submitted - in whole or part, to another university for the award of any other degree.

Signature:

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Summary

Defence against predators is fundamental to increasing an organism's fitness. My thesis explores this central theme in behavioural ecology using stingless bees as study organisms. The thesis contains a general introduction (Chapter 1), three data chapters (2-4) and a final discussion (5).

Chapter 2 is a comparative study of aggression in nest defence among stingless bee species, and describes a new form of nest defence, suicidal biting, which is most prevalent in the genus *Trigona.*

Chapter 3 describes a remarkable behaviour in *Partamona helleri*, which crashes head-first when entering its nest. An experiment suggests that this behaviour helps to avoid predation at the nest entrance.

Chapter 4 studies nest defence in the hovering guards of *Tetragonisca angustula*, and demonstrates that through coordinated vigilance, a group level behaviour rarely observed in animals, the ability of the group to detect predators is enhanced.

Publications arising from this thesis

Shackleton, K., Toufailia, H.A., Balfour, N.J., Nascimento, F.S., Alves, D.A., Ratnieks, F.L.W. (2015) Appetite for self-destruction: suicidal biting as a nest defense strategy in *Trigona* stingless bees. Behavioral Ecology and Sociobiology 69, 273–281. (Chapter 2).

Shackleton, K., Alves, D.A., Ratnieks, F.L.W. Spatial organisation enhances collective vigilance in the hovering guards of *Tetragonisca angustula* stingless bees. Behavioral Ecology (accepted). (Chapter 4).

Other papers published during my PhD

Shackleton, K., Ratnieks, F.L.W. (2016) Garden varieties: How attractive are recommended garden plants to butterflies? Journal of Insect Conservation 20, 141–148.

Ratnieks, F.L.W., Shackleton, K. (2015) Does the waggle dance help honey bees to forage at greater distances than expected for their body size? Frontiers in Ecology and Evolution 3, 31.

Shackleton, K., Balfour, N.J., Al Toufailia, H., Gaioski, R., de Matos Barbosa, M., Silva C.A. de S., Bento, J.M.S., Alves, D.A., Ratnieks, F.L.W. (2016) Quality versus quantity: Foraging decisions in the honeybee (*Apis mellifera scutellata*) feeding on wildflower nectar and fruit juice. Ecology and Evolution 6, 7156–7165.

To be submitted

Shackleton, K., Balfour, N.J., Al Toufailia, H., Alves, D.A., Ratnieks, F.L.W. Unique nest entrance structure of *Partamona helleri* stingless bees leads to remarkable 'crash landing' predator avoidance behaviour. (Chapter 3).

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Contents

Chapter 1 – General introduction

1.1 General background to predator defence

Defending oneself against predators is key for survival, and has obvious fitness benefits. As such, predator defence is a central theme in behavioural ecology. In the predator-prey arms race, prey that live in groups may be able to evolve defensive adaptations that take advantage of group-living (Dawkins & Krebs 1979, Krebs & Davies 1993).

While groups of animals may be a more conspicuous target than lone individuals (Watt et al. 1997), group-living confers several advantages in avoiding predation. With more eyes on the lookout, groups have a greater ability to detect predators than have lone individuals, and can then alert their neighbours to the threat (Pulliam 1973, Lima 1995). The prey group may respond by mobbing the predator, forcing it to retreat (Dugatkin & Godin 1992, Ostreiher 2003, Graw & Manser 2007). When the prey chooses to flee, groups may use movement patterns to confuse predators and evade capture (Landeau & Terborgh 1986, Hogan et al. 2017). Because predators are likely to capture only one or two individuals, the probability of an individual being targeted by a predator is diluted by living in a group, known as "selfish herding" (Hamilton 1971, Watt et al. 1997). Furthermore, individuals may also use the presence of conspecifics as indicators of safe foraging patches leading to aggregations (Dawson & Chittka 2014).

As well as defending oneself, defending offspring or kin offers indirect, but potentially large, fitness benefits (Hamilton 1964). The research in this thesis will address questions in social defence using study systems in the stingless bees. Stingless bees are eusocial insects that build and live in a nest containing their kin and valuable food stores. The worker bees often go to extreme lengths to defend themselves and their nests from predators and parasites, and have evolved many remarkable defensive behaviours. Because the stingless bees are social animals, this thesis considers both the individual and collective behaviours involved in defence.

1.2 Study species – the stingless bees

1.2.1 Diversity and distribution

The stingless bees (tribe: Meliponini, family: Apidae) are a diverse group of highly eusocial bees, with over 500 described species and many more undescribed (Wille 1983, Michener 2000). The stingless bees are distributed throughout the tropics and southern subtropics, however, their diversity is concentrated in the neotropics (Camargo & Pedro 1992). As their name suggests, the stinger, ancestral in aculeate Hymenoptera, is vestigial in stingless bees (Kerr & de Lello 1962).

1.2.2 Colony structure

The stingless bee colony typically consists of a single queen, which is singly mated, and her daughter workers (Peters et al. 1999). As a eusocial group, there is a reproductive division of labour and overlapping generations with the queen performing the majority of the colony's reproduction and the workers helping to rear the offspring (Wilson 1971, Palmer et al. 2002). The workers perform a variety of tasks and transition between tasks through a phenomenon known as the age-related division of labour, or age polyethism (Seeley 1982, Sommeijer 1984, Traniello & Rosengaus 1997). In this way, the colony increases productivity by allocating the workers with the lowest remaining life expectancy (or residual value) to perform the most risky tasks (Traniello & Rosengaus 1997). The young workers perform low risk tasks inside the nest such as tending the queen and provisioning brood cells, whereas the older workers perform more risky tasks outside of the nest such as guarding and foraging (Seeley 1982). There is considerable variation in colony size among species, from a few hundred workers in some *Melipona* spp. to over 10,000 in *Trigona* spp. (Wille 1983).

Like most bees, stingless bees are central place foragers (Orians & Pearson 1979). The foragers travel between the nest and food patches, collecting nectar and pollen, although other feeding habits include collecting meat from animal carcasses, fruit or robbing other colonies (Roubik 1982, Sakagami et al. 1993, Noll et al. 1996). Many species have the ability to communicate the location of food sites to nestmates, although the mechanisms are perhaps less sophisticated than the waggle dance of the honey bee (Nieh & Roubik 1995, Nieh & Roubik 1998, Nieh 2004).

1.2.3 Nest structure

Stingless bee colonies live in a nest, typically a perennial, long-lived structure built by the bees themselves. There is considerable variation in nesting habits, even among closely related species (Roubik 2006). The nest is often located in a tree cavity or crevice, but may also be located in rocks, underground, built into an active termite nest, or be exposed on a substrate such as a tree or wall (Roubik 1989, Roubik 2006). The main nesting material is cerumen, a mixture of waxes and resins, but may also include plant fibres, mud, vertebrate faeces and small stones (Roubik 1989, Roubik 2006). The nest is connected to the outside world by a distinct entrance, often elaborated into a tube, which shows considerable variation in its external structure (Figure 1.1, 1.2) and may run for many centimetres internally. At the centre of the colony is the brood chamber, which contains hexagonal brood cells that are often arranged into layers of horizontal comb (Wille 1983, Figure 1.1). However, cells in some species are not arranged in combs but in clusters and are spherical rather than hexagonal. The brood chamber is surrounded by a sheath of cerumen known as the involucrum, and above and below this are areas of food storage. Food is stored in roughly spherical or ovoid storage pots, and separate pots are used for honey and pollen. The internal structure of the nest may also be supported by vertical pillars. The bees build batumen plates at the limit of the nest made of cerumen and mud, which may cover the whole outer surface in the case of exposed nests (Wille 1983).

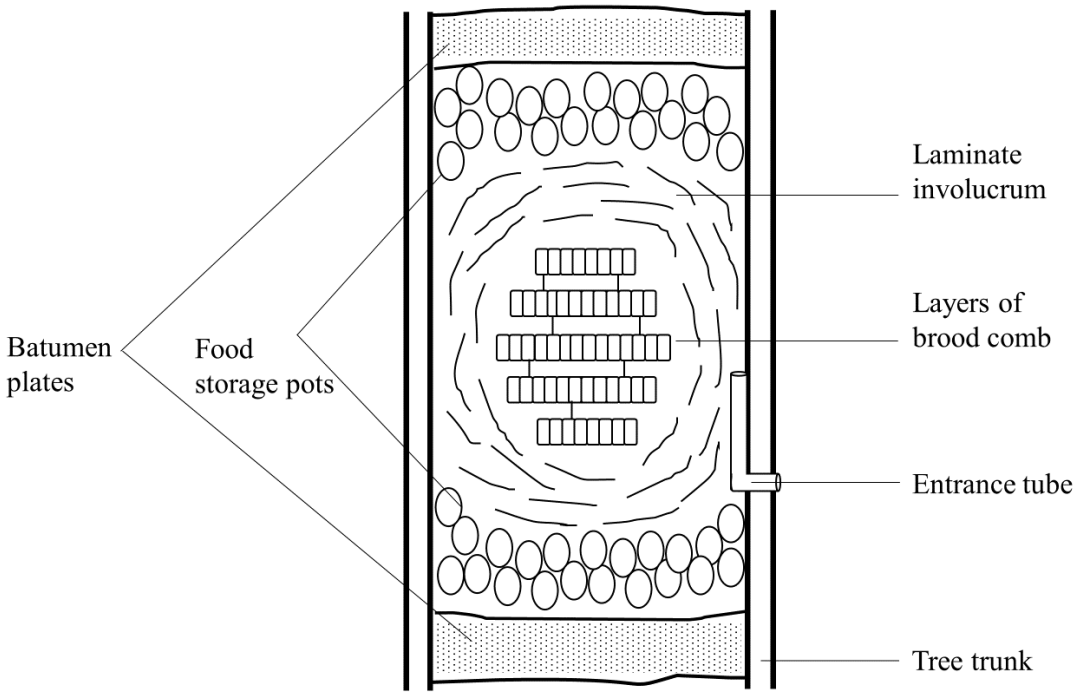

Figure 1.1: Basic design of a stingless bee nest, built inside a tree cavity with the main features shown. Redrawn from Wille (1983).

1.*2.4 The nest entrance*

One of the most important areas of the nest is its entrance. The entrance represents the interface between the colony and outside world, and is the location at which the colony and individuals are likely to be attacked. There is considerable variation in entrance structure among species (Figure 1.2). The simplest entrances are a small hole, with no external architecture. More elaborate entrances consist of long tubes built of wax, resin and mud, and may incorporate various secondary structures for defence (section 1.6).

The most fundamental feature of the nest entrance is its size, which again varies greatly among species and has implications for both foraging and defence. Large entrances can accomodate greater forager traffic, but more guards are required for their defence (Couvillon et al. 2008a). The most narrow of entrances allow only one or two bees to pass at once and are defended by a single guard (for example, many *Melipona* spp. and *Friesomelitta* spp. nests). The largest entrance belongs to *Trigona silvestriana*, which builds a tube up to a metre long and 10 centimetres in diameter (Roubik 2006).

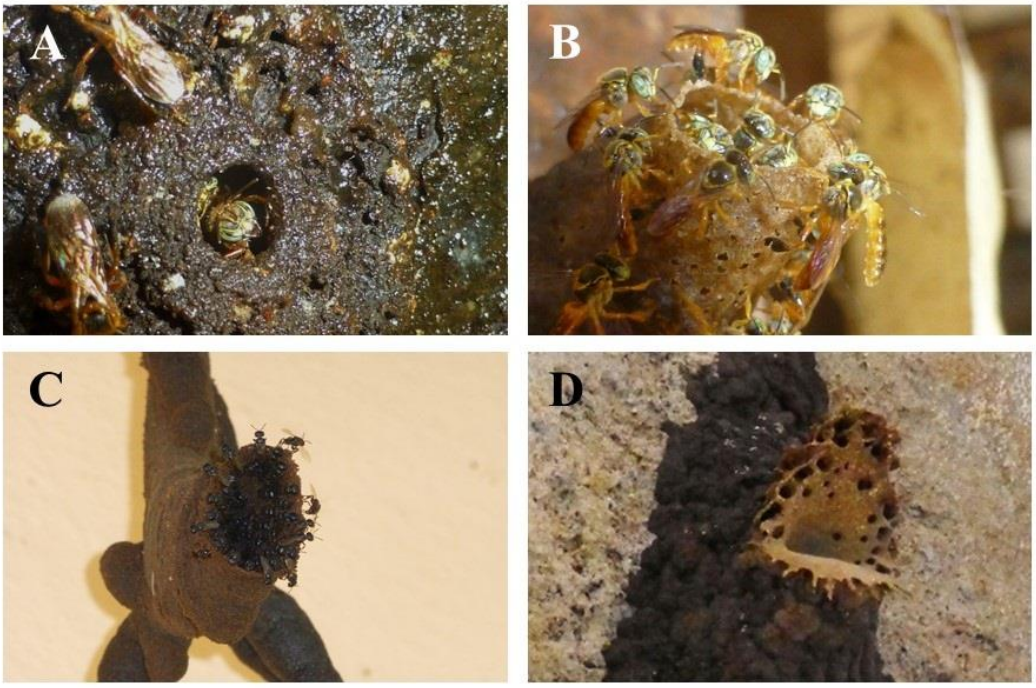

Figure 1.2: The variation in the nest entrances of stingless bees (Meliponini). **A** Narrow entrance of *Friesomelitta varia*, **B** tubular entrance of *Tetragonisca angustula*, **C** tubular entrance of the obligate robber bee *Lestrimelitta limao*, **D** funnel-shaped entrance of *Partamona helleri* (outer entrance shown).

1.2.5 Why study stingless bees?

Stingless bees are an immensely useful group for behavioural ecology research, yet are relatively understudied compared with *Apis* and *Bombus*. The unique and often undescribed behaviours exhibited by stingless bees make them a rich source for new discoveries and the description of natural history. One advantage of stingless bees over *Apis* and *Bombus* is that their diversity lends them to comparative studies. This is particularly pertinent for studies on defence because stingless bees show great variation in their aggression towards intruders, guard number, colony size, and defensive mechanisms and abilites. In the neotropics, it is relatively easy to locate wild colonies of the more common species. At the field sites used in this thesis in São Paulo State, Brazil, a day's exploration may yield several wild colonies of *Partamona helleri*, *Scaptotrigona depilis*, *Tetragona clavipes*, *Tetragonisca angustula*, and *Trigona*

hyalinata. Last, like *Apis*, many species of stingless bees can be kept in hives, making them easier to use and observe for research.

1.3 Who are the enemies of stingless bees?

Because stingless bee nests contain such a dense concentration of nutritionally valuable resources (honey, pollen and brood), they represent an attractive target for many potential predators.

1.3.1 Vertebrate predators

Medium to large sized mammals are a particular threat to colonies because of their ability to excavate a nest and kill a whole colony in a single attack (Roubik 1989, Figure 1.3A). In South and Central America, the tayra *Eira barbara* (Mustelidae) is known as the "honey robber" and can excavate both ground and tree nests (Posey & Camargo 1985). Other expert excavators include the giant armadillo *Priodontes maximus* and *Tamandua* spp. (Kerr 1969), while more generalist predators such as the spectacled bear *Tremarctos ornatus* and skunks (Mephitidae) can also attack and kill nests (Caron 1978, Roubik 1989).

Smaller vertebrates may wait outside a colony and ambush individual bees as they enter and leave the nest. Malagodi et al. (1986) noted that toads (*Bufo* spp.) could consume many bees at the nest entrance when it was in an accessible location. Birds have little problem accessing arboreal nests and woodpeckers, for example, can kill small bee colonies on their own (Gerling et al. 1983). I have also observed a video of a *Tropidurus* spp. lizard ambushing *Partamona helleri* workers at the nest entrance (Zacaris 2015, Figure 1.3B).

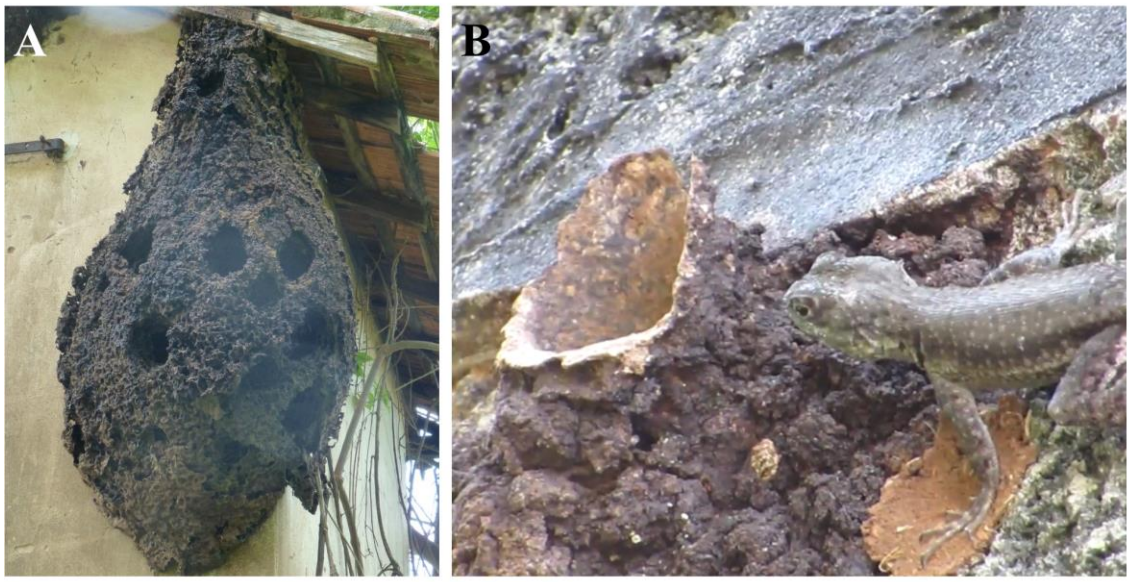

Figure 1.3: Vertebrate predation on stingless bee nests. **A** Evidence of a large mammal having attacked and killed a *Trigona hyalinata* nest. **B** *Tropidurus* spp. lizard perched outside a *P. helleri* nest and ambushing workers as they leave or return. This photo is a still from a Youtube video (Zacaris 2015).

1.3.2 Social insect predators and parasites

Other social insects are probably a more significant natural enemy of stingless bees than vertebrates. Ants are one of the major predatory groups of other insects in the tropics and attack stingless bee nests (Hölldobler & Wilson 1990). However, the greatest enemies of stingless bees are perhaps other stingless bee colonies.

Inter-colony conflicts in stingless bees may occur both within and among species. A literature review by Grüter et al. (2016) revealed that at least 60 species engage in heterospecific conflicts. The primary motivator for a colony to attack another is not predation but rather the theft of resources or cleptobiosis, also known as "robbing". Independent acquisition of resources is a time consuming and costly process, and it may therefore, be more profitable for a colony to steal the resource of another (Ribbands 1949, Visscher & Dukas 1997, Iyengar 2008). Because stingless bee robbers and their hosts share a common biology, the resources collected and stored by the hosts (for example honey, resin, wax) are directly comparable to those desired by the robbers.

Indeed, victim colonies may be chosen based on the quality of honey they have stored (Grüter et al. 2016).

The attack by one colony on another is known as a "raid". Raids can be highly variable in their duration (hours to weeks), the retaliatory aggression displayed by the victim colony, and the damage inflicted on both sides. A low level of aggression may occur where victim colonies are unable to mount an effective defence, and they instead reduce adult mortality by declining to fight (Roubik 1989). An extreme level of aggression occurs between Australian *Tetragonula hockingsi* and *T. carbonaria*, where the former attacks the latter not to rob but to usurp the whole colony (Cunningham et al. 2014). These species form huge fighting swarms that may leave thousands of workers dead (Cunningham et al. 2014). If the attacking colony is successful, the old colony is evicted and a new queen and the workers of the victor are installed.

In most species, robbing appears to be facultative and may occur when floral resources are scarce (honey bees; Downs & Ratnieks 2000, stingless bees; Grüter et al. 2016). For example, *Melipona fuliginosa* acquires the majority of its resources from flowers but is an occasional robber of honey bees and other *Melipona* colonies (Roubik 1983). However, some species have evolved to be obligate robbers, and no longer visit flowers for resources (Michener 1974, Wittman et al. 1990, Sakagami et al. 1993). One of the most notable obligate robbers is *Lestrimelitta limao*, which is known to raid a variety of species, including those larger than itself (Sakagami et al. 1993, Grüter et al. 2016).

Sakagami et al. (1993) give detailed descriptions of *L. limao* raids on host colonies: Raids begin with only one or a few scouts at the nest entrance of the host colony. However, within minutes dozens more robbers arrive at the nest entrance and use their tenacious fighting ability and a chemical signal to overwhelm the defenders. The robbers secrete large amounts of citral from their mandibular glands, which acts as a recruitment signal for their nestmates, and overpowers the olfactory nestmate recognition cues of the host colony (section 1.4.3). The robbers use their powerful mandibles to attack guard bees and can force much larger bees such as *Melipona* spp. or *Apis mellifera* to retreat by attacking the mandibles, joints or sting. Once the entrance

has been subjugated, the robbers form a guard ring preventing workers of the host colony from returning, and aggressively attack any insects flying in proximity of the nest. Some minutes later, *L. limao* workers enter the food and brood storage areas of the nest and begin to pillage. The duration of the raid is highly variable, ranging from hours to weeks.

Raids have obvious fitness consequences for the host colony. The robbers will deplete food stores and empty the food provisions in brood cells, and young larvae and eggs are killed (Sakagami et al. 1993). Some food stores, particularly pollen, are not completely depleted and neither are nesting materials such as resin. Adult mortality of the host colony is often low, as workers retreat to areas of the nest that are of no interest to the robbers. However, where the hosts mount an aggressive response, both sides may lose hundreds of workers. Small stingless bee host colonies may be eliminated, while *Apis mellifera* colonies have been known to vacate their hive or nest following a raid. Furthermore, host defences are reduced and colonies are left vulnerable to other natural enemies. Sakagami et al. (1993) noted that following a *L. limao* raid, parasitic phorid flies quickly invaded a colony which resulted in its death. The risk of starvation is also presumably increased.

The pressure of robbing by *Lestrimelitta* is so great that it has lead to the independent evolution of specialised defenders in several other stingless bee species (Grüter et al. 2017a), notably in *Tetragonisca angustula* (Grüter et al. 2012). At an ecological scale, the threat of robbing also leads to an upregulation of a colony's investment in defenders (Segers et al. 2016).

1.3.3 Individual level predation

While predation at the colony level is probably the greater selective pressure, predation of individual workers outside the nest can result in significant losses to the colony workforce. Visscher & Dukas (1997) found that foraging honey bees outside the nest had a constant mortality rate. Predation may occur while foraging, as workers may become ensnared in the webs of orb weaver spiders or captured on a flower by a crab spider. However, in this thesis I will focus on ambush predation at the nest entrance.

The nest entrance is a choke point with high levels of worker activity, and is thus an attractive location for predators to lie in wait. I have already discussed how small vertebrates can inflict significant losses on a colony (section 1.3.1). However invertebrates may also ambush stingless bees at the nest entrance. Assassin bugs (Reduviidae) can kill enough foragers to kill a colony, and some species may specialise on bees, living from egg to adult on the outer surface of a nest (Johnson 1983, Roubik 1989). I have also observed many jumping spiders (Salticidae), lurking on the entrance tubes of stingless bee nests, waiting to strike on any bees that stray from the safety of the entrance hole.

For completeness, it should be stated that the stingless bees face a near inexhaustible variety of other threats, including parasitoid wasps, wax moths, nematodes, phoretic mites, and pathogens. Fascinating though they are, the modes of defence against these threats, such as hygienic behaviour (Al Toufailia et al. 2016), are beyond the scope of this thesis.

1.4 Threat detection

1.4.1 Vigilance behaviours

Before an animal can mount a defensive response against a potential threat, the threat must first be detected and identified. Vigilance behaviours are commonly used by animals in order to detect threats such as predators (Pulliam 1973), and vigilance is a well-studied topic in behavioural ecology. Vigilance involves the prey individual actively scanning its environment for predators. In the classic studies on vigilance in birds and mammals, the behavioural response is for the animal to raise its head and scan the environment (Pulliam et al. 1982). If the animal detects a predator it can then react, for example by fleeing, and avoid being eaten. However, performing a vigilance behaviour also bears a cost, because time spent on vigilance is time not spent foraging (Illius & Fitzgibbon 1994). Indeed, vigilance is often studied in the context of foraging animals, and time spent feeding is often used as a proxy measure in vigilance studies (Lima 1988). That is, time spent not feeding is considered to be time spent vigilant.

In social animals, perhaps the most important factor influencing vigilance is the size of the group. The group size effect has been observed in many birds and mammals, and as a general rule, as group size increases individual vigilance decreases (Bertram 1980, Elgar & Caterall 1981, Lima 1995). There are two main explanations for the group size effect, the many eyes hypothesis and the dilution effect.

The many eyes hypothesis holds that as group size increases, the probability that at least one group member will be scanning at any one time also increases (Lima 1995, Fairbanks & Dobson 2007). This means that in large groups, the overall level of group vigilance can be 100 percent. When a predator is detected, the rest of the group can then be alerted to its presence and escape. Therefore, each individual can spend greater time feeding while benefiting from greater group vigilance. For example, Bertram's (1980) study of ostrich vigilance against lion predators found that in larger groups each bird scanned less but the probability of at least one bird scanning at any one time increased slightly. Furthermore, Kenward (1978) showed that large flocks of pigeons were able to detect their goshawk predators at greater distances, and that the hawks had lower hunt success when targeting a larger flock.

In the dilution effect, the risk of an individual being targeted by a predator is reduced in larger groups, because there are more group members from which to choose (Dehn 1990, Roberts 1996). The probability of an individual being targeted can thus be summarised as 1/n, where n is the number of individuals in the group. This assumes that the predator will only capture a single prey individual, and that a larger, more conspicuous group will not suffer so many more attacks, as to counteract the reduced probability of the individual being targeted within the group. This may lead to selfish herd effects, where group members may vie for the safest positions within the group (Hamilton 1971, King et al. 2012).

How do these rules relate to social insects? In the above cases, group members act independently and selfishly with respect to each other, scanning less and occupying the best positions at the expense of their neighbours. There seems to be little incentive for group members to cooperate and coordinate their vigilance, perhaps because the

individual fitness benefits are lower than the costs of doing so. However, in a group with high levels of relatedness, such as a social insect colony, there are obvious incentives for group members both to coordinate and to act altruistically. Indeed, social insect workers have already largely sacrificed their direct fitness, and the guards (section 1.5.1) are already tasked with defending the nest. Some vertebrates, such as meerkats and mongooses, operate a sentinel system where one individual looks out for the group (Rasa 1986). However, even here, the sentinels may act selfishly, only performing vigilance behaviours if it is in their best interest, such as when it is already satiated and guarding is the optimal activity to increase its own fitness (Clutton-Brock et al. 1999). Last, vigilance in social insects differs from many other animals because the guards defend a fixed location (the nest), and options to flee are limited.

Chapter 4 uses the unique hovering guards of *Tetragonisca angustula* (section 1.5.2), to test for coordinated vigilance and its effect on predator detection in a eusocial insect.

1.4.2 Proximate methods of predator detection in social insects

One common mode used by prey to detect predators is through vision (Hartman & Abrahams 2000, Amo et al. 2004, Martin & Piersma 2009). The compound eye of bees and other insects is fundamentally different from the vertebrate eye. The compound eye is composed of many individual ommatidia, each with a light gathering lens at the cuticle (Land & Nilsson 2012). The lenses form the facets on the surface of the eye. Below each lens is a cone, bordered by pigment cells, and below each cone are elongate nerve cells that transform the gathered light into nerve signals (Chapman 1982).

The resolution of the compound eye is determined by the angle between adjacent ommatidia (the interommatidial angle). Larger insects have larger eyes containing more ommatidia (thus a smaller interommatidial angle) and larger facet lenses, both of which yield a greater resolution. However, lens size is tiny compared with a vertebrate eye such that the resolution of a compound eye is far lower. An eye of a medium sized bee with a facet lens diameter of 25μm is able to resolve two objects with an angular separation of 1°, equivalent to seeing in pixels the size of a fingernail at arm's length (Chapman 1982, Land & Nilsson 2012). This resolving power is about 60 times less

than that of a human, and the resolving power of smaller bees is even poorer (Jander & Jander 2002, Land & Nilsson 2012). In the context of detecting a predator, a bee may not have too much trouble distinguishing the shape of a vertebrate from a moderate distance but would have difficulty in detecting another bee until nearby.

The compound eyes of many insects are relatively large such that they cover a greater proportion of the head surface area than the human eye (Seidl & Kaiser 1981, Merry et al. 2006). This gives the insect a wide visual field. For example, honey bees have a horizontal visual field extending 313° (Seidl & Kaiser 1981). However, insects still have an area of dead space (a blind spot) in the centre of the posterior hemisphere of the head (Seidl & Kaiser 1981, Merry et al. 2006). That is, they cannot see directly behind them. Furthermore, the density of ommatidia varies across the surface of the eye (Land 1997, Land & Nilsson 2012), such that at the periphery, towards the rear, resolution is decreased. This indicates that the ability of an insect to detect a predator is lower if the predator approaches from the rear.

A far greater proportion of communication in bee societies occurs through chemical signals and olfaction. The chemical receptors found on the antennae play an important role in the olfaction and contact chemoreception (Chapman 1982). The surface of an antenna is covered in thousands of microscopic sensory structures including hairs, pits and plates (Winston 1987). *Melipona* bees were found to have 13 types of sensilla on their antennae (Ravaiano et al. 2014). These structures give bees an olfactory sense 10 to 100 times more acute than that of humans for odours that are biologically relevant to the bee (Ribbands 1955, Winston 1987). Where the visual discriminatory ability of bees may fall short, their acute olfactory and contact chemoreception abilities enable them to accurately recognise intruders.

1.4.3 Nestmate recognition

Despite lacking great visual acuity, the ability for social insects to distinguish nestmates from non-nestmates, known as nestmate recognition, is widespread (Butler & Free 1952, Hölldobbler & Wilson 1990, Suka & Inoue 1993, Gamboa et al. 1996). The nest entrance is the hub of nestmate recognition, because it is here where the colony

interfaces with the outside world and is the point of attack for many intruders. Guards orient and touch their antennae, forelegs and tongue on the incomer to discern its origin (Butler & Free 1952, Moore et al. 1987). Incomers deemed to be a nestmate are allowed to pass unhindered into the nest whereas those deemed to be foreign intruders are met with aggression. In stingless bees, aggression takes the form of biting, grappling and the use of defensive chemicals (section 1.6).

In theory, guard bees could use any sensory modality to discriminate between nestmates and non-nestmates, but it practice it is done through the similarity between the incomer's odour and that of the colony (Breed et al. 1988). This forms the basis of the acceptance threshold model (Reeve 1989). Under the model, incomers with olfactory cues that are above some threshold of dissimilarity from the colony odour are rejected, whereas those below the threshold are accepted into the colony. However, bees from different colonies will overlap somewhat in their odour cues. Two bees from the same colony will differ little in their odour, conspecifics from different colonies will differ somewhat more, heterospecific bees will differ to a greater degree, and so forth. This leads to the potential for error in two directions: non-nestmates may be allowed to enter while nestmates may be rejected.

Bees are able to adjust their acceptance threshold in response to threat of robbing and predation (Downs & Ratnieks 2000). When the threat is high, guards are less permissive and *vice versa*. The threshold can be adjusted over the course of a season (Downs & Ratnieks 2000) or shift rapidly over a matter of minutes (Couvillon et al. 2008b).

1.5 Defenders of the fortress

1.5.1 Guards

One of the tasks a worker bee may transition through in the age-related division of labour is that of a guard. As their name suggests, these bees function to guard the nest, and are usually stationed at the nest entrance with their heads and mandibles pointed outwards, ready to intercept any intruders. Guards tend to be an older subset of the workers, as guarding is a risky task, but foragers are older still (Seeley 1982, Hölldobler & Wilson 1990). Guard behaviour involves the inspection of other bees entering the

nest (nestmate recognition, section 1.4.3), and responding to intruders when they are encountered. Defensive behaviours are diverse and include fighting, biting, harassment, and the use of chemicals to injure the intruder and recruit additional defenders (section 1.6).

In stingless bee species with larger nest entrances, guards are usually seen forming an outward facing ring around the lip of the entrance (e.g. *Trigona*, *Scaptotrigona*, *Lestrimelitta*, Figure 1.2C). In species with narrow entrances, only a single guard may be visible, often blocking the whole area of the entrance (e.g. *Melipona*, *Friesomelitta*, Figure 1.2A). However, additional defenders may be stationed behind, further down the entrance tube.

1.5.2 Specialised defenders – soldiers

Many species of social insect employ an even more specialised subset of the workers for colony defence. Different from age polyethism, in many species the workers are divided into physically distinct castes, with considerable variation in size, shape, function and behaviour. These physical castes may be distributed in size along a continuous scale, fit into discrete size classes, of use some combination of the two depending on the species (Hölldobler & Wilson 1990). The larger workers are known as majors (or sometimes super majors) and when morphologically or behaviourally predisposed for defence and fighting, they are known as soldiers.

A soldier caste has evolved independently multiple times in the social insects, and soldiers are known in many species of ant, termite, aphid and thrips. Soldiers are best known in the ants, where extreme variation in worker size can be seen. In Asian marauder ants (*Pheidologeton diversus*) and the leafcutter ants (*Atta* spp. and *Acromyrmex* spp.), the difference in mass between the largest and smallest workers can be over two orders of magnitude (Wilson 1980, Hölldobler & Wilson 1990). In leafcutter ants, the huge size differentiation among workers is driven by diet and the need to gather and process leaves and tend the fungus garden, as well as by defence, and the largest workers are soldiers (Hölldobler & Wilson 1990).

A common feature of soldiers is an allometrically large head, which is better able to support large mandibles and powerful adductor muscles for biting. The mandibles are typically used to cut and shear enemies (in the case of leafcutter ants) or may be adapted for piercing as in the sickle shaped mandibles of army ant soldiers (Hölldobler & Wilson 1990). A more unusual method of biting defence occurs in *Orectognathus versicolor* ants, where the soldiers spread their elongate mandibles wide before snapping shut on an intruder with sufficient force to pinch and "bounce" it away (Carlin 1981). In some species the enlarged head is used to block the nest entrance. *Colobopsis* spp. soldiers have a large, flattened head which forms a tight-fitting plug over the narrow nest entrance (Wilson 1974). When a nestmate needs to pass the soldier pulls back to open the way. These defensive morphological specialisations of soldiers can make soldiers ill adapted for other tasks. For example, Délye (1957) noted that *Cataglyphis bombycina* soldiers, which possess long, sharp mandibles, perform poorly at carrying items around the nest and often have to be transported around by other workers.

Until recently, ants were thought to be the only hymenopterans to produce a soldier caste, as soldiers were unknown in the bees and wasps. This may be due to the existing developmental constraint of the larvae in bees and wasps being reared in cells of a fixed, predetermined size. However, the first instance of a soldier in a bee was discovered in *Tetragonisca angustula* stingless bees by Grüter et al. (2012).

Soldiers in *T. angustula* do not exhibit the extreme levels of size polymorphism or allometry typically seen in ant soldiers. However, worker size is still bimodally distributed as soldiers are approximately 30 % larger than foragers with little overlap in size (Grüter et al. 2012). Guards also have allometrically larger heads and smaller legs compared to foragers. These size differences translate into improved fighting performance, as larger soldiers are better able to hold their own against a *L. limao* worker (a foe three times larger) before being defeated (Grüter et al. 2012). Furthermore, guards are better at recognising intruders than smaller workers, by virtue of a greater number of antennal olfactory receptors (Grüter et al. 2017b). *Tetragonisca angustula* soldiers not only guard the nest, but also have a greater task variety than other

workers, including building, maintenance and waste disposal, and they transition faster through such age related tasks. *Tetragonisca angustula* have thus been described as an "elite" sub caste of workers (Hammel et al. 2016).

It has more recently been discovered that a soldier caste has evolved multiple times within the stingless bees. (Grüter et al. 2017a). However, most species do not show the same bimodality in size between guards and foragers seen in *T. angustula*. This repeated evolution of soldiers is thought to have been driven by robbing pressure from *Lestrimelitta* (section 1.3.2).

Most stingless bee guards are stationed just within the entrance tube or on its outer lip if one is present (Figure 1.2). However, *Tetragonisca angustula* bees possess a unique defence in the form of guards that hover near the entrance, typically within a few centimetres (Wittman 1985, Kärcher & Ratnieks 2009, Grüter et al. 2011). Hovering guards are also present in the closely related *T. fiebrigi* (Christoph Grüter, pers comm), but are far less studied than *T. angustula*. These hovering guards are able to track incoming bees and intercept intruders before they reach the nest entrance (Wittman 1985). This provides an advantage over species without hovering guards, which can only detect and react to intruders when they reach the nest entrance (van Zweden et al. 2011). Furthermore, the greater the number of hovering guards, the greater the defensive 'perimeter' is from the nest entrance (van Zweden et al. 2011).

1.6. Natural history aspects of nest defence

Among the defensive responses of stingless bees, those in the genus *Trigona* are known locally in Brazil for being the most aggressive. *Trigona* nests are among the largest of the stingless bees, possessing many thousands of workers (Wille 1983). Even a human standing innocuously near a nest is liable to receive an attack from these bees. Furthermore, *Trigona* can also be aggressive in the defence of floral resources (Johnson & Hubbell 1974). At the nest, a *Trigona* defensive response is straightforward, as they do not make use of sticky resins or caustic chemicals. Rather, many individuals swarm the intruder in a mass attack. The workers particularly target the head area of the intruder, and may continue to bite and harass the victim long after it has left the vicinity

of the nest. It has been noted that *Trigona* may perform a kind of 'death grip' behaviour, where they clamp the mandibles onto the victim and refuse to let go (Buchwald & Breed 2005, Shorter & Rueppell 2012, section 1.7).

Defensive responses are often associated with the release of alarm pheromones by the defenders. These chemicals serve to alert nestmates to the presence of an intruder. Pheromones may also be deposited on the intruder to direct nestmates to its location. In species that nest in aggregations, alarm pheromones may also alert and recruit neighbouring colonies to join in the defence. Other defensive chemicals may be directed towards the intruders. Bees in the genus *Oxytrigona* possess enlarged mandibular glands that produce a caustic secretion causing blisters on mammalian skin for up to several days after contact (Wille 1983). *Melipona rufiventris* guards secrete a sticky white substance from their mandibles when agitated, while *Tetragona clavipes* carry sticky resins on their hind legs that can used to immobilise arthropod intruders (Kerr & Lello 1962, personal observations). In response to an attack on the colony, stingless bees may also upregulate their collection of defensive materials such as resin (Leonhardt & Blüthgen 2009).

The structure of the nest entrance itself is of great importance in nest defence. First, a small size makes the entrance more defensible (Couvillon et al. 2008a). Closing the entrance at night to protect against nocturnal predators is a common behaviour in social insects. This is seen for example in *T. angustula*, which folds its wax entrance tube shut each night (Grüter et al. 2011). In addition to this pre-emptive closure of the entrance, some species have developed mechanisms to block the entrance rapidly in response to an intruder. *Melipona flavolineata* store balls of batumen to the side of the internal entrance tube (Nunes et al. 2014). When the nest is exposed to the odour of robber bees, ball is rolled to the entrance to close it off to intruders (Nunes et al. 2014). Upon being attacked by robber bees, *Hypotrigona braunsi* pours honey down its entrance tube (Portugal-Araújo 1958). The adaptive benefits of this behaviour are not completely understood, because it kills both robbers and nestmates caught in the flow and the honey is taken by the robbers.

Some species may also build secondary defensive structures at the nest entrance. False entrances and false nests are a common feature of many species. These structures serve to distract and delay intruders, and divert them from the real nest. A false entrance may lead to a dead end, while a false nest may contain a chamber containing empty storage pots (Kerr 1969). These can be effective against enemies both large and small. Kerr (1969) reported that *Tamandua* may abandon its excavation upon encountering a false nest due the apparent lack of reward. Camargo & Pedro (2003) reported that in a *L. limao* raid on a *Partamona vicina* nest, the robbers occupied a false nest and failed to find the true nest before giving up.

1.7 Self-sacrificial defence

Guarding the nest is an inherently risky behaviour, as there is an increased likelihood of encountering and engaging enemies, and many workers may lose their lives. However, a more extreme form of defence involves the self-sacrifice of workers in defence of their colony (Shorter & Rueppell 2012). Self-sacrificial defence may be distinguished from general defensive behaviour in that self-sacrificial behaviour leads to the inevitable (or near inevitable) death of the worker. While social insect workers have already sacrificed their direct fitness, workers are still valuable to the colony, such that their loss decreases colony fitness and their lives should not be sacrificed heedlessly. Self-sacrificial behaviours should arise and persist when their contribution to colony fitness exceeds that of the worker's non-self-sacrificial defensive effort plus the worker's future contribution to the colony.

Self-sacrificial behaviour has evolved multiple times in the social insects, and is generally associated with the defence of the nest (Shorter & Rueppell 2012). These behaviours are often paired with and aided by morphological adaptations that increase the severity of the defensive response and guarantee the death of the worker. The most familiar example of self-sacrificial defence is that of sting autotomy (self amputation) in the honey bee (Hermann 1971). Sting autotomy has also evolved independently in ants and wasps (Hermann 1971, Sledge et al. 2002), and all have similar morphological adaptations. The honey bee sting has many barbs that cause it to become embedded in the flesh of the victim. The sting connects to the venom gland, which is only connected

to the rest of the abdomen by weak musculature. When the bee pulls away, the sting apparatus easily tears from the body, leaving the sting and venom sack lodged in the victim. The ruptured body wall of the bee leads to its death some hours later. However, the venom gland continues to pump and deliver venom to the victim, as well as release alarm pheromones that may alert nestmates to the presence of an intruder. A similar mechanism to autotomy is sting avulsion, found in *Vespula maculifrons* wasps (Greene et al. 2012). Here, the sting becomes embedded in the flesh but does not separate from the wasp, leaving the wasp itself stuck in the victim.

Another widespread form of self-sacrificial defence is autothysis, which occurs in *Camponotus* spp. ants and several genera of termites (Shorter & Rueppell 2012. The process involves the defender rupturing its own body wall to release a chemical substance onto the attacker. The chemical composition varies in composition among taxa, but all have comparable effects. Substances are generally caustic, causing direct damage to the victim, and congeal when exposed to air, entangling the appendages of arthropod victims (Jones et al. 2004). Autothysis probably evolved in response to arthropod predators, as the substances can easily kill or at least disable an insect victim but have little effect on larger vertebrates (Maschwitz & Maschwitz 1974). In *Camponotus* spp., enlarged mandibular glands span the length of the body (Shorter & Rueppell 2012). The ants contract their abdomen until the body wall ruptures, at which point the contents of the glands are spread over the immediate area, potentially consuming more than one victim and affording the ants a numerical advantage in combat. The ant may also climb onto the face of the victim before autothysing for maximum effect (Davidson et al. 2011).

The proximal morphological mechanisms in termites differ somewhat from ants, but the behaviours of defenders and functional effects of the chemicals are similar. In *Globitermes sulphurous* and *Apilitermes longiceps* the rupture occurs at the head and the released substance congeals around the victim (Deligne & De Coninck 2006). In *Glossotermes oculatus* the secretion appears not to target the attackers directly, but rather blocks the nest galleries to prevent attackers from passing (Šobotník et al. 2010).

Gall inhabiting *Quadrartus yoshinomiyai* aphids also use a chemical based mode of self-sacrifice. When an intruder threatens their colony, older, post-reproductive *Q. yoshinomiyai* individuals secrete a sticky, waxy substance from their cornicles onto the predator (Uematsu et al. 2007). This immobilises the intruder but also binds the pair together, which results in the death of the defender.

So far, these methods of self-sacrifice may be described as "instantaneous" (Shorter & Rueppell 2012). That is, active engagement of an intruder. However, pre-emptive self-sacrifice, that is, self-sacrifice to prevent a potential future attack, also occurs. Nest entrance closure at night is common in social insects (Kerr & Lello 1962, Grüter et al. 2011), as it protects against attack from nocturnal predators. However, in *Forelius pusillus* ants, a few (1-8) workers remain outside as the entrance is closed (Tofilski et al. 2008). These workers do not try to enter, but rather aid in the closure and concealment of the nest entrance from the outside. Come morning, these workers are dead, either blown away by the wind or eaten by predators (Tofilski et al. 2008).

Host suicide is another form of self-sacrificial defence, which occurs when an individual is infected with a contagious pathogen (Smith-Trail 1980, Rueppell et al. 2010). If an infected worker removes itself from the colony to die alone, it reduces the chance of the pathogen spreading to its nestmates. Individuals should remove themselves if the cost of remaining, in terms of infecting nestmates, exceed the benefits of continuing to work until death. For example, *Temnothorax unifasciatus* ant workers that are dying from fungal infection remove themselves from the colony and cease all interactions with their nestmates, preventing further infection (Heinze & Walter 2010). The authors also found that this was not due to an enforced eviction by uninfected nestmates or manipulation of the host by the fungal pathogen, but rather altruistic self removal by the infected individual.

Despite being a widespread and speciose group of eusocial insects, which is also subject to attack from many sources, there are no definitive records of self-sacrificial behaviour in the stingless bees. However, there have been several informal records of self-sacrificial behaviour, most notably in the genus *Trigona*. Buchwald & Breed (2005)

noted that in staged combats between *Trigona fulviventris* individuals, bees would often bite and grapple, and would rather die than disengage from their opponent. Johnson & Hubbell (1974) studied aggression among stingless bees under the context of resource competition and also noted that *Trigona* foragers would grapple and may "never separate alive". I observed such a behaviour first hand between *Trigona* of two different species foraging at a tree wound (Figure 1.4). Bees that engage each other either in flight or on vegetation bite each other, then fall to the ground where they continue to grapple. Both refuse to disengage and both eventually die.

A further example of this "suicidal biting" can be seen in *Tetragonisca angustula*. As a relatively small species, *T. angustula* cannot hope to directly overpower and kill the intruders that may attempt to attack or rob its nest, which may be an order of magnitude or greater in mass. However, *T. angustula* guards employ a strategy of clamping their mandibles on the wings of intruders, effectively disabling them (Grüter et al. 2012). This results in the eventual death of both combatants, as the *T. angustula* worker refuses to disengage and will die clamped to the victims wing. Meanwhile the intruder is left unable to fly and may never return to its colony. On the floor surrounding *T. angustula* nests it is common to observe workers of other bee species in futile attempts to fly, because a dead *T. angustula* worker, or only its head, is clamped onto the wing of the victim (Figure 4.1B).

A formal study of defensive self-sacrifice in stingless bees in presented in Chapter 3, with particular emphasis on the biting defence of *Trigona* spp.

Figure 1.4: Two *Trigona* spp. workers of different species (unidentified). Both individuals were foraging from a tree wound but attacked each other, presumably for the purpose of resource defence. The bees fell to the ground fighting and died together, with neither willing to release its "death grip".

1.8 Individual level defence

Even social insects must defend themselves individually when they venture out from the colony. Insects are a hugely diverse group and have evolved a wide variety of anti-predator behaviours. Defences may be chemical, by being foul-tasting or by emitting a toxic substance as in the bombardier beetle (Eisner 1958, Brower 1958, Arndt et al. 2015), by stinging in the case of many Hymenoptera (Hermann 1971), or by mimicking an insect with a potent defence to trick predators (Brower 1958, Nishikawa et al. 2015). Visual or acoustic displays may act to startle or confuse the predator, and may even mimic a larger predator in order to cause hesitation (Oloffson et al. 2010, Oloffson et al. 2012, Oloffson et al. 2013). Many insects employ a more passive defence in crypsis, to avoid detection entirely (Vallin et al. 2006, Wiklund et al. 2008). However, by far one of the most widespread defences across the animal kingdom is by fleeing.

Fleeing may involve the prey attempting to outpace, outlast, or outmanoeuvre the predator in a pursuit (Guinet et al. 2007, Domenici et al. 2008, Wilson et al. 2018), or dart quickly into a refuge that the predator is unable to reach (Kramer & Bonenfant 1997). In the case of eusocial bees, that refuge is commonly the nest. However, the nest entrance is also a choke point with high levels of worker activity and is thus an attractive location for ambush predators to wait at (Mackay 1982, Schatz & Wcislo 1999). Even for a large bee colony, losing individual workers to ambush predation is not trivial, as predation pressure can be high enough to reduce colony productivity and even lead to colony death in extreme cases (Johnson 1983). Chapter 3 explores how *Partamona helleri* stingless bees combine their unique nest entrance structure with an individual level behaviour of returning workers to provide a novel twist on the classic anti-predator flight response.

Chapter 2 – Appetite for self-destruction: suicidal biting as a nest defence strategy in *Trigona* stingless bees

2.1 Abstract

Self-sacrificial behaviour represents an extreme and relatively uncommon form of altruism in worker insects. It can occur however, when inclusive fitness benefits are high, such as when defending the nest. We studied nest defence behaviours in stingless bees, which live in eusocial colonies subject to predation. We introduced a target flag to nest entrances to elicit defensive responses, and quantified four measures of defensivity in 12 stingless bee species in São Paulo State, Brazil. These included three *Trigona* species, which are locally known for their aggression. Species varied significantly in their attack probability (cross species range = 0-1, $P<0.001$), attack latency (7.0-23.5 seconds, $P=0.002$), biting duration of individual bees (3.5-508.7 seconds, $P<0.001$) and number of attackers (1.0-10.8, $P<0.001$). A "suicide" bioassay on the six most aggressive species determined the proportion of workers willing to suffer fatal damage rather than disengage from an intruder. All six species had at least some suicidal individuals (7-83%, $P<0.001$), reaching 83% in *T. hyalinata*. Microscopic examination revealed that all three *Trigona* had five sharp teeth per mandible, a possible defensive adaptation. Suicidal defence via biting is a new example of self-sacrificial altruism and has both parallels and differences with other self-sacrificial worker insects, such as the honey bee. Our results indicate that suicidal biting may be a widespread defence strategy in stingless bees, but it is not universal.

2.2 Introduction

Behaviours enhancing self-preservation, such as predator defence, are fundamental to survival (Alcock 2005). Similarly, parental defence of offspring is widespread despite the increased risk of parental mortality, as it increases defender's total reproduction (Andersson et al. 1980, Klemperer 1982, Sefc et al. 2008, Nazareth & Machado 2010). Social insect workers, which typically have no direct reproduction, use a variety of strategies to defend their nests against predators. In extreme cases this involves the self-sacrifice of defenders (Shorter & Ruepell 2012). Social insect nests are worth defending as they contain not only offspring (brood), but also the reproductive individuals, food

stores and nesting material, while the nest itself is often a valuable resource (Seeley 1985, Roubik 2006). Natural selection will favour defensive self-sacrifice in worker insects if this increases their inclusive fitness more than non-suicidal defensive strategies.

Suicidal defence has evolved multiple times in social insect workers and takes various forms. Sting autotomy, which is well known in the honey bee (*Apis mellifera*), involves the self-amputation of the sting apparatus from the body. This increases venom delivery, releases alarm pheromone, and the apparatus can continue to pulsate long after the stinging event (Hermann 1971, Burrell & Smith 1995). Autothysis, the rupturing of the body wall to release defensive chemicals is known in ants (for example, *Camponotus* spp., Maschwitz & Maschwitz 1974, Davidson et al. 2011) and termites (for example, *Globitermes* spp., Bordereau et al. 1997). A similar mechanism has been described in aphids, which produce a sticky secretion causing the defending aphid to adhere to the predator, thereby immobilizing it (Uematsu et al. 2010). All of these strategies combine a behavioural component with morphological adaptations which inevitably cause mortality in the defending workers. Worker self-sacrifice however, need not require morphological specializations. For example, worker *Forelius pusillus* ants have a form of pre-emptive suicidal defensive behaviour (Tofilski et al. 2008). Workers seal their nest entrances from the outside in the evening, resulting in most dying before the entrance is reopened from the inside in the morning.

Stingless bees (Meliponinae) comprise many hundred described species, are found worldwide in the tropics, are closely related to honey bees, and live in perennial eusocial colonies of c. 100 – 100,000 workers (Michener 2000, Roubik 2006). As their name suggests, stingless bees are unable to sting as the stinger is vestigial (Michener 2000). However, they still face predation at the nest from many sources ranging from mammals to nest-robbing bees (Wille 1983, Suka & Inoue 1993, Roubik 2006). Defence therefore, is important for colony survival. Despite lacking a sting, stingless bees possess numerous defensive mechanisms including biting, harassment, caustic chemicals, alarm pheromones and hovering guards (Kerr & de Lello 1962, van Zweden et al. 2011). Observations of *Trigona* spp. stingless bees in Brazil indicate that humans

standing in the vicinity of nests are invariably attacked (personal observations). These *Trigona* workers give painful and persistent bites, are difficult to dislodge, and frequently die in the attack. Buchwald & Breed (2005) also noted grappling and biting behaviour in *Trigona* in conflicts with other bees, where individuals would refuse to disengage from each other resulting in death. Individuals inevitably die in colony defence, but if biting workers are more willing to die than disengage from an intruder, it would constitute suicidal behaviour.

Suicidal biting in stingless bees has not been formally reported in the literature (Shorter & Rueppell 2012). If it does occur, it would be a novel form of self-destructive behaviour. This study aimed to determine whether the intense biting that we have casually experienced in *Trigona* bees is so extreme as to justify being considered a form of suicidal defence. We carried out a field study of three *Trigona* species in São Paulo State, Brazil, that personal experience had indicated are candidates. We studied nine further stingless bee species to put the *Trigona* results in a wider context. Our results show that workers of all three *Trigona* and three of the other nine species bit a target "intruder" so persistently and tenaciously that a significant proportion suffered fatal physical damage.

2.3 Methods

Study sites and species

The study was conducted in São Paulo State, Brazil at two locations. Most stingless bee colonies were located on the campus of the University of São Paulo at Ribeirão Preto. The remainder were c. 50km away at Fazenda Aretuzina, a farm near the town of São Simão dedicated to wildlife conservation and stingless bee research. All field data were collected between 08:00 and 17:35 on sunny days in March 2014 at temperatures of 25-35C.

A total of 12 species were studied (Table 2.1). We aimed to study at least three colonies of each species but for two species, *Trigona fuscipennis* and *T. spinipes*, we were only able to locate two colonies of each (Table 2.1). Previous experience indicated that colonies of *Tetragonisca angustula* were highly variable in their aggression towards

perceived threats. Therefore, 10 colonies of this species were studied. The majority of colonies were kept in hives within apiaries, but some were wild, nesting in trees or on buildings (Table 2.1). All hive-dwelling colonies had modified their nest entrances using wax and resin to construct their 'natural' entrance structures.

Table 2.1: List of the 12 stingless bee study species, colony locations, number of colonies used, number of flag tests performed and number of bees biting the flags.

Species	**Colony locations***	**Bee nesting sites**	**Typical colony size (no. foragers)**	**Number of colonies**	**Number of flag tests**	**Number of biting bees**
Trigona hyalinata	USP, FA	Buildings, trees	Large	5	28	255
Trigona fuscipennis	USP	Trees	Large	2	14	104
Trigona spinipes	USP	Trees	Large	2	14	146
Partamona helleri	USP	Buildings, trees, apiaries	Small	4	20	129
Scaptotrigona depilis	USP	Apiaries	Medium	5	30	109
Tetragona clavipes	USP	Trees, apiaries	Medium	7	30	68
Tetragonisca angustula	USP	Apiaries	Small	10	40	38
Frieseomelitta varia	USP	Apiaries	Small	5	30	10
Melipona scutellaris	USP	Apiaries	Very small	5	30	0
Melipona quadrifasciata	USP	Apiaries	Very small	5	30	6
Melipona rufiventris	FA	Apiaries	Very small	5	15	0
Leurotrigona muelleri	USP	Tree stumps, apiaries	Very small	4	20	0

* USP = University of São Paulo at Ribeirão Preto, FA = Fazenda Aretuzina near São Simão

Defensivity bioassays

We performed two field bioassays to quantify aggression and suicidal behaviour. To induce bee colonies into attacking, we used black felt flags, 10 × 10 cm mounted on poles, as used in previous research on honey bee defensive behaviour (Hunt et al. 1998). In a flag test, the flag was waved within 5 cm of a colony entrance for a period of one minute or until the bees attacked, whichever was sooner. An attack was defined as one or more bees leaving the nest entrance, landing on the flag and proceeding to bite. If an attack occurred, we recorded the time at which it began (latency) following the start of the flag test and then carefully removed the flag to a distance of 5-10 m from the colony. We then recorded the number of bees biting and the duration of attack for each

bee, i.e. the time from the start of the attack at which each individual bee left the flag. An individual bee's attack was deemed to have ended when the bee left the vicinity of the flag (bees would occasionally leave the flag but return seconds later). Additionally, for each species we calculated "overall aggression" as a descriptive measure defined as $\frac{PND}{L}$, where P = mean probability of attack, N = mean number of bees, D = mean $\log_{10}$ attack duration and L = mean $\log_{10}$ latency. Fresh flags were used following each attack to exclude the effect of any previously deposited alarm pheromones. We tried to study the same colony no more than twice on a single day but in a few cases this was not possible. On one occasion, we also tested flags of different colours including blue, white, and a multi-coloured flag of green, blue, yellow and white, but none of these flags elicited a measurable defensive response.

A suicide bioassay was performed only on species which, in the flag tests, had a probability of attack >0.5 and a mean attack duration >15 seconds. Bees attacked a flag as before. The flag was then removed to a distance of 20 m from the nest. Biting bees were subjected to two levels of the bioassay in order to test their degree of self-sacrifice. Firstly, bees were brushed for five seconds using a 5mm width paintbrush which caused no physical harm. Secondly, remaining bees had their wings on both sides clamped using a pair of forceps. Forceps have been used previously to induce suicidal responses in *Camponotus* ants (Maschwitz & Maschwitz 1974). Bees were pulled until they either let go of the flag and could subsequently fly away when released, or suffered damage to the wing to the point that they could no longer fly when released. The damage usually consisted of large portions of the wing membrane being removed or a whole wing separating at the thorax. Occasionally however, the bee's body would separate between the first and second thoracic segments leaving the mandibles, head and first thoracic segment clamped to the flag. Since these bees could no longer fly and return to their nests they were deemed to have suffered fatal damage, and therefore were self-sacrificing. Although the flag material was not an exact representation of any particular predator, we simply wanted to give the bees a choice between disengaging or continuing to bite the flag. We repeated this for 30 bees of each species (Table 2.1).

Mandibular Teeth

Using a stereomicroscope, we photographed the mandibles of each study species in order to identify any characteristics which might aid in defence such as size and teeth.

Controlling for colony size

We wanted to allow for colony size in our analysis so we used incoming worker traffic as a proxy (Couvillon et al. 2008a). Each colony in the study had its nest entrance video-recorded for a period of three minutes between 09:00 – 12:00 when foraging activity was high. The number of foragers returning to the colony was then counted from the video.

Statistical analysis

For the flag test bioassay we used mixed-effects models to fit attack probability (binomial error structure), number of bees per attack (Poisson error structure), attack latency and attack duration (both $\log_{10}$-transformed) as response variables. Flag nested within colony was fitted as a random effect for attack duration, as we measured duration for multiple bees per flag and sampled each colony with multiple flags. Colony was fitted as a random effect for the other response variables. We also controlled for time of day, worker traffic, and attack number, as it is possible that the more attacks a colony receives the more aggressive it may become (Couvillon et al. 2008b). The maximum models were fitted then simplified to the minimum adequate models through backwards elimination of non-significant terms and model comparison using ANOVA (Crawley 2013). For the suicide bioassay, we fitted the degree of self-sacrifice as the response in a mixed-effects model (alive or dead, binomial errors) with species as the explanatory variable and colony as a random effect. We present test statistics and P-values of our minimum adequate models compared to the null models using ANOVA. All analyses were conducted using R version 3.1.0 and the R packages lme4 and nlme (Bates et al. 2013, Pinheiro et al. 2013, R Core Team 2014).

2.4 Results

We studied a total of 59 colonies making 302 flag tests that resulted in 868 bees biting the flags. There was considerable variation between bee species in the levels of all measures of defensive behaviour. Bee species differed significantly in their likelihood to attack the flag ($P < 0.001$, $\chi^2 = 114.47$, D.F = 11, Figure 2.1A) and fell into three broad categories. Three species (*Leurotrigona muelleri, Melipona quadrifasciata, and M. rufiventris*) did not respond aggressively at all. Rather, the guards always stayed within the entrance and often retreated further inside when provoked by the flag. This indicated that guards perceived the flag as a potential threat, but chose not to attack. Five had an intermediate response (for example, *Tetragonisca angustula*) where guards would usually leave the entrance when provoked but did not always attack the flag. Four species (all *Trigona* species and *Partamona helleri*) were extremely aggressive and always attacked the flag.

Of the nine species that did attack, the number of bees that bit the flag varied significantly among species ($P < 0.001$, $\chi^2 = 65.80$, D.F. = 8, Figure 2.1B) ranging from an average of 7.8±1.1 – 10.8±0.99 (means ± standard error) bees in the three *Trigona* species, with the maximum of 10.8 for *T. spinipes*, to 1 in *M. scutellaris*, a 10-fold difference. The maximum number in a single flag test was 22 bees from a *T. hyalinata* colony. The non-zero minimum of one bee occurred consistently in *M. scutellaris*. This species attacked the flag only 20% of the time (Figure 2.1A) but when it did just one bee bit the flag (mean = 1±0, Figure 2.1B). This species has a narrow entrance hole that normally has a single guard present, blocking most of it (Couvillon et al. 2008a).

Species also varied significantly in the latency of attack with a three-fold difference (range = 7.0±0.75 – 23.5±4.0, P = 0.002, likelihood ratio = 24.06, D.F. = 8, Figure 2.1C) and duration of attacks with over a 100-fold difference (range = 3.5±1.1 – 508.7±59.7, P < 0.001, likelihood ratio = 221.58, D.F. = 8, Figure 2.1D). The three *Trigona* species attacked with the shortest latencies (7.0±0.7 – 7.9±1.9 seconds) and longest durations (157±12.4 – 509±59.7 seconds). The longest single bee attack duration was in *T. fuscipennis* at 51m45s. Figure 2.1E shows a combined "overall aggression" showing the more aggressive nature of *Trigona* versus the other species.

Of those species that did attack, all did so with mean latencies of < 24 s, indicating that the flag waving period of one minute was enough to provoke any colony likely to attack into attacking. Six species; *Tetragona clavipes, Scaptotrigona depilis, Partamona helleri, Trigona fuscipennis, T. hyalinata,* and *T. spinipes,* met the threshold of an attack probability > 0.5 and mean attack duration > 15 s and were used in the suicide bioassay.

In the suicide bioassay, the proportion of self-sacrificial individuals differed significantly with species ($P < 0.001$, $\chi^2 = 19.267$, D.F. = 5, Figure 2.1F), but all species that bit the flag had at least some individuals willing to suffer fatal damage rather than disengage. Suicidal individuals were observed to clamp their mandibles into the flag and their refusal to relinquish their grip resulted in the fatal damage. *Trigona* spp. had the highest mortality, 33-83%. *Trigona hyalinata* was especially suicidal with 83% of individuals being pulled apart by the forceps rather than letting go. This was both over twice as high as the next highest species *T. fuscipennis,* and was the only species where the proportion of suicidal individuals was greater than 50%.

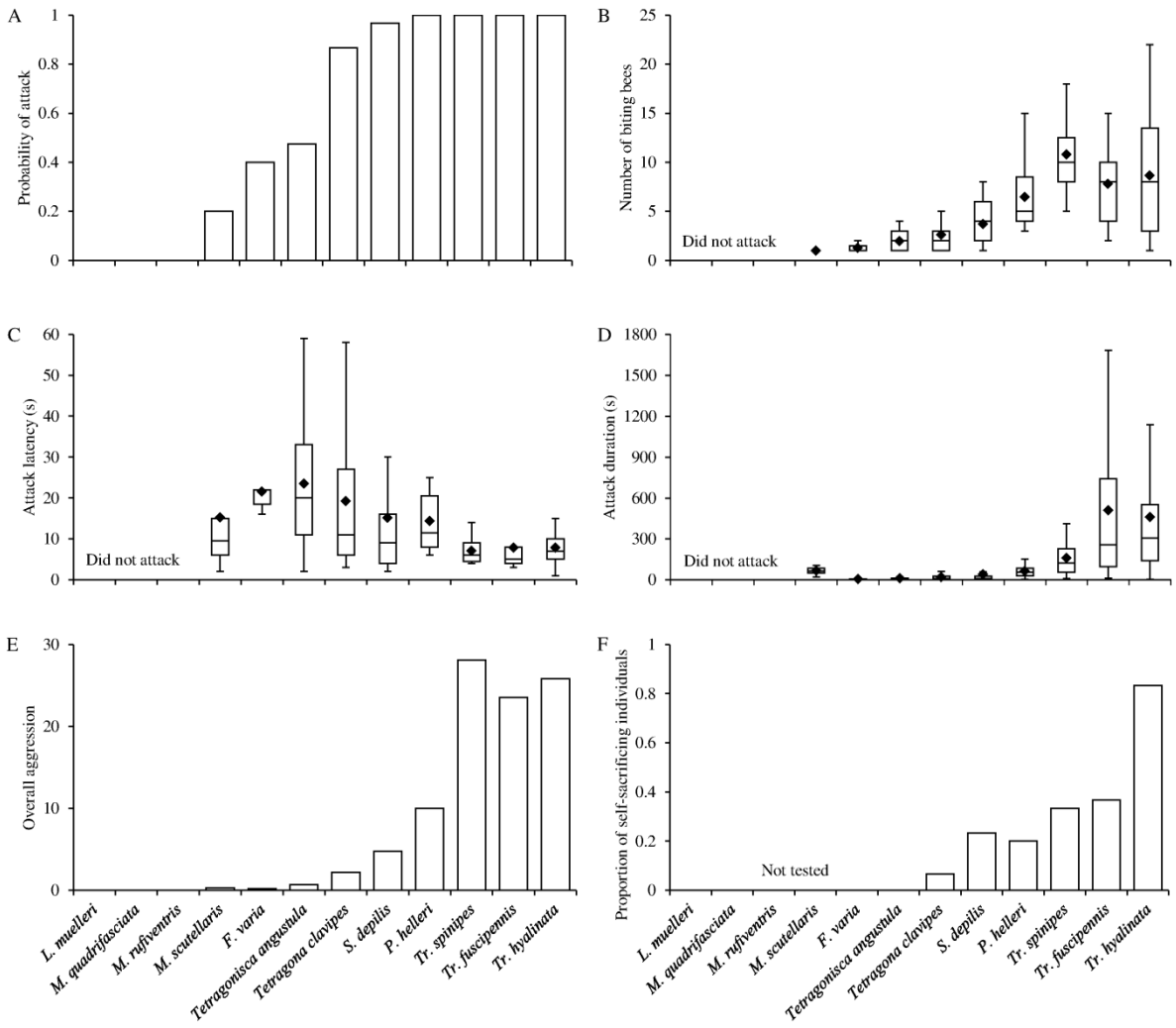

Figure 2.1: Variation in four measures of aggression in 12 stingless bee species in the flag test bioassay: A) Probability of at least one bee from a colony biting the flag. B) Number of biting bees per flag test. C) Time until attacking the flag (latency). D) Duration individual bees attacked the flag for. E) A combined measure of overall aggression. F) The proportion of self-sacrificial individuals in the suicide bioassay. Whiskers 1.5×interquartile range, means shown as diamonds.

The mandible photographs show that some species, particularly the three *Trigona*, have sharp teeth (Figure 2.2). By comparison, the mandible of a worker honey bee *Apis mellifera* is toothless and spoon-like in shape, a morphology shared by some of our study species such as the three *Melipona*. Some species such as *Tetragona clavipes*, which was moderately aggressive in our bioassays, had two small teeth on the basal part

of each mandible which was otherwise spoon-shaped. All species of *Trigona* possessed serrated mandibles with five large teeth.

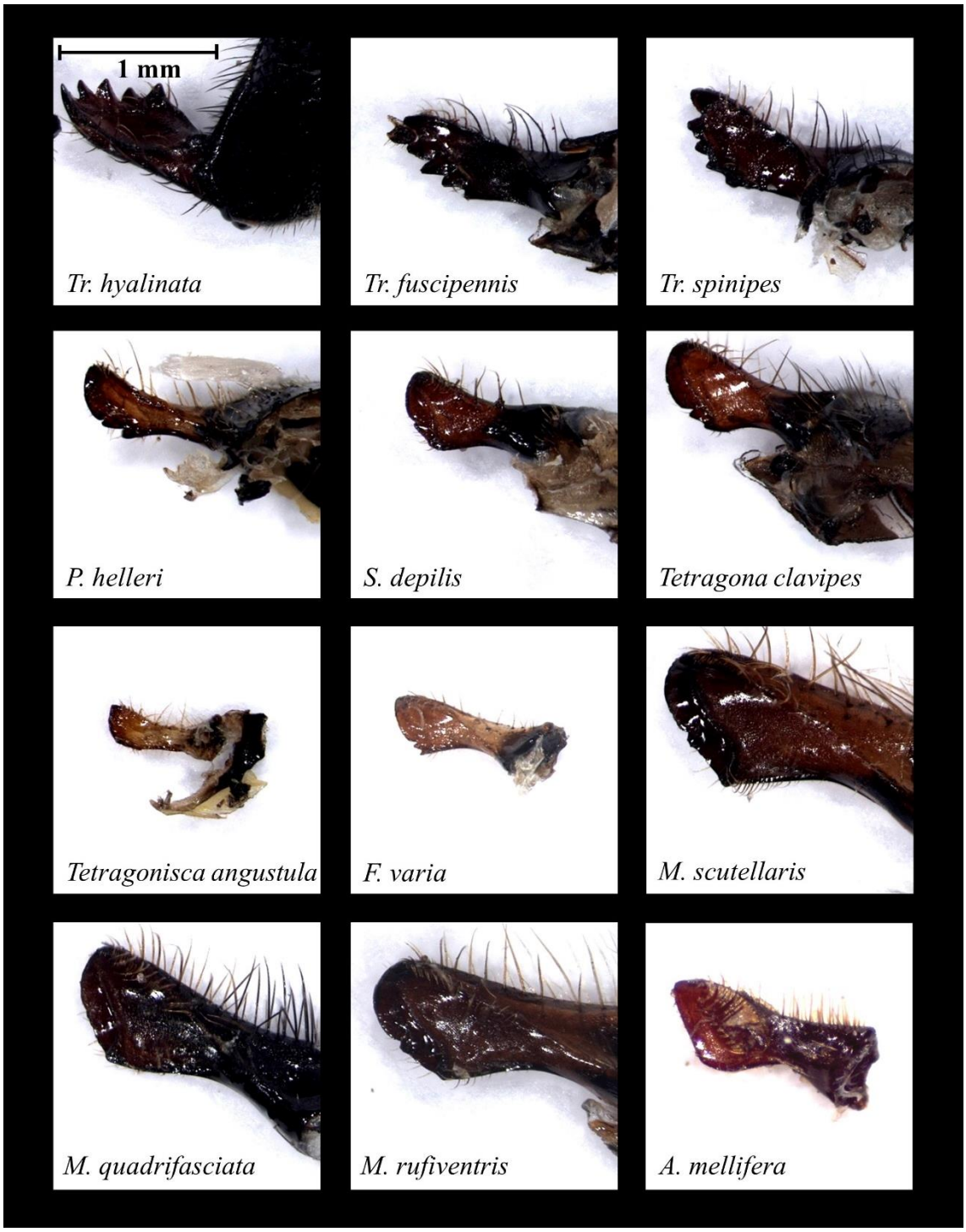

Figure 2.2: Photographs of mandibles from 11 stingless bee species and the honey bee (*Apis mellifera*) for comparison, showing the presence of teeth, particularly on *Trigona*. All pictures to same scale.

2.5 Discussion

Our study shows that suicidal biting as an anti-predator defence occurs in stingless bees. We believe this is the first clear demonstration of suicidal biting in defence of the nest by any insect worker. The results support our general impression from casual observations, that *Trigona* are particularly defensive and even suicidal. The three *Trigona* species led the nine other species in all four aggression measures in the flag test, and had the largest proportion of self-sacrificial individuals in the suicide bioassay. The most suicidal was *T. hyalinata* in which almost all the bees tested (83%) suffered fatal damage rather than disengage their mandibles from the flag. However, self-sacrifice was not confined to *Trigona* as it occurred in a significant proportion (7-23%) of the test bees in the three other species submitted to the suicide bioassay (*Partamona helleri, Scaptotrigona depilis, Tetragona clavipes*). This represents a new example of convergent evolution with other suicidal insect workers. Because levels of within-species self-sacrifice may be low, suicidal behaviour may be difficult to detect and therefore, potentially more widespread than previously thought. However, our results make it clear that not all stingless bees have suicidal biting. In fact, three of our study species did not attack the target flag at all.

High levels of aggression and suicidal behavior should both enhance nest defence. Bees that attack more often, in greater numbers, with shorter latencies, and for longer durations will presumably be more effective at repelling the current attack and deterring potential future attacks (Schmidt 1990). Furthermore, in committing self-sacrifice through their jaw clamping behaviour, stingless bees can immobilize or kill intruding insects (Grüter et al. 2012) and cause longer-lasting pain to vertebrate predators, preventing further attack on their colonies.

Higher levels of colony defence are likely to increase both colony survival and the mortality risk to the defender. Natural selection should therefore, favour an optimal level of defence, where risk to the colony is traded-off against the future value of the defender to the colony (Andersson et al. 1980). The optimal level of defence should increase with colony size, because the colony contains more kin and is thus of greater

value. In social insects, colony size can be large, 10,000s of individuals in *Trigona* for example (Roubik 2006), while the reproductive value of workers is low. Furthermore, many social insects exhibit age polyethism, where the risky tasks such as guarding are performed by the older workers with shorter life expectancies (Tofilski 2002). These factors can lead to a very high investment in defence and potentially, the decision to commit self-sacrifice (de Catanzaro 1986, Brown et al. 1999). Eusocial insects could thus be described as having an exaptation for self-sacrificial behaviour.

In social insects, each additional worker adds proportionally less to colony fitness (Michener 1964, Nonacs 1991). The relative costs of sacrificing workers will thus be less in large colonies than small ones. Stingless bee species vary greatly in colony size (Wille 1983). A suicidal attack of 20 bees from a 10,000 strong *Trigona* colony versus a 100 strong *Melipona* colony represents a loss of 0.2% vs 20% of the total worker population. In small colonies, the costs of mass attacks, especially those involving suicidal behaviour, would likely be greater than the benefits from improved defence. Our results support this theory, as the most aggressive and self-sacrificial species in the study (*Trigona*) were those with the largest colonies (Roubik 2006). Attacking intruders singly does not represent an effective defensive strategy, because the pain and damage per bite, while unpleasant, is unlikely to drive an intruder away. Mass attacks are therefore required to offer a more robust defence. A formal analysis of colony size and aggression is not within the scope of this paper, as only 12 species were studied of which suicidal behaviour was only observed in six. This does however, raise two important questions: First, is the mean colony size of a species a good predictor of aggression and self-sacrificial behaviour? Second, is there intraspecific plasticity in aggression and self-sacrifice between small and large colonies?

Close examination of the mandibles revealed that the *Trigona* species possessed serrated mandibles bearing sharp teeth. This morphological specialization may be a defensive adaptation, allowing *Trigona* to cause more pain and damage to intruders. Mandibular teeth however, may have also evolved in response to selective pressures other than colony defence. Stingless bees use a variety of materials to construct their nests including resin and soil (Wille 1983), the collection and manipulation of which

may be aided by toothed mandibles. *Trigona* mandibles are similar in appearance to those of mason bees (for example, *Osmia bicornis*: Megachilidae) that excavate soil from their nests, and reminiscent of the fossorial forelegs of mole crickets (Gryllotalpidae). However, *Partamona helleri* nests are composed largely of soil but this species possesses only a single small tooth on each mandible. *Trigona* are also known to aggressively defend foraging patches against other bees, using their mandibles to harass, bite and kill competitors (Johnson & Hubbell 1974, Nagamitsu & Inoue 1997). The vulture bee *T. hypogea* feeds on carrion and fruit in place of pollen and nectar, and mandibular teeth may facilitate foraging on such alternative food sources (Roubik 1982). While the *Trigona* species in the present study are not obligatory necrophagous, Wille (1983) suggested that they may turn to carrion when pollen sources are scarce.

Although the serrated mandibles of *Trigona* are a morphological feature that almost certainly enhances the effectiveness of their biting defence, how it interacts with the tendency to self-sacrifice is unclear. Suicidal biting differs from most previously known examples of self-sacrifice in worker insects in lacking a morphological mechanism that guarantees mortality. Honey bee sting autotomy and autothysis in ants and termites nearly always result in the death of the worker (Hermann 1971, Shorter & Rueppell 2012), whereas the stingless bees in our study showed a gradation in suicidal behaviour and in all but *T. hyalinata*, mortality was less than 50%. However, bees with more serrated teeth may be more likely to become entangled in the hair or integument of the victim, and suffer fatal damage when pulled away, even if their behavioural response would be to release their grip. On balance, it seems that self-sacrificial biting is more a behavioural than morphological phenomenon. In our bioassay bees were often so engrossed in their attack on the flag that they did not attempt to evade the brush or forceps.

Non-aggressive stingless bee species should not be thought of as defenceless, as biting is only one of a wide variety of defensive adaptations (Kerr & de Lello 1962). For example, when provoked during the flag tests, guards from the non-aggressive species in our study retreated from the nest entrance rather than confront the flag. These species

tended to be those with very small entrances relative to their body sizes (Couvillon et al. 2008a). This strategy represents the opposite of a mass attack, where intruders must combat guards singly in a narrow space. Several species in our study, most notably the mildly aggressive *F. varia* and moderately aggressive *Tetragona clavipes* frequently deposited sticky, odorous resins on the flag. While this behaviour would have little effect on a vertebrate predator, it is likely very effective at immobilizing other stingless bees, and may be similar in function to secretions found commonly in ants and termites (Prestwich 1979, Bordereau et al. 1997, Davidson et al. 2011).

Killing or disabling intruders is especially important in defending the nest against robbing by other stingless bees, as allowing scout robber bees to successfully scout can result in mass attacks on the colony and potentially far greater costs than the loss of a few suicidal workers. This is paralleled by honey bee colony defence against the Asian giant hornet *Vespa mandarinia* (Ono et al. 1995). Biting defence is seen in the conflicts between one of our study species, *Tetragonisca angustula,* and the obligate robber bee *Lestrimelitta limao*. Despite a large size disadvantage, *T. angustula* guards are able to clamp onto the wings of *L. limao* for long durations. This prevents the robber from flying and returning to its own colony, but often results in the death of the *T. angustula* worker (Grüter et al. 2012).

Our study has shown a wide range in the aggressive, defensive behaviour of stingless bees. The presence of suicidal defensive biting in half our study species indicates that this behaviour is potentially a widespread defensive strategy. In our experience, the three *Trigona* study species almost invariably will attack any human standing within a few meters of a nest entrance, often within seconds. Workers attack the head but also other parts of the body. So tenacious and unpleasant is the attack, that the victim is forced into a hasty retreat. Bees are especially difficult to remove from hair and if a bee is removed and released, it usually resumes its attack on the head immediately. The only recourse for the victim therefore, is to flee and kill the bees to stop the attack.

Methodological comments

A number of methodological improvements could be made to this study, and could enhance future studies of defence in social insects. The application of the flag treatment was not controlled in that it was performed by a human who could have been influenced by the attacking bees. We were as consistent as possible when designing the study to follow a defined flag waving protocol, but future studies could use an automated device or a metronome to standardise flag waving. Had the timing of flag presentation been consistent (i.e. were it always presented for one minute and not ceased when an attack occurred), some results may have differed. Attack probability and latency would not have changed. The range of values for attack duration may have changed, but the mean value should not be affected. However, in species that attacked with lower latency, those aggressive species would have had the opportunity to commit more bees to the attack. Removal of the flag following an attack would therefore, lead to an underestimate for the number of attackers. However, this would be unlikely to change our general conclusions, and if anything our impression that that *Trigona* spp. are extremely aggressive may have been reinforced. In the suicide test, the flag was used only to collect bees for use in the brush and forceps assay, so did not influence the results.

In an ideal study, we would have studied more colonies for certain species (such as *T. fuscipennis* and *T. spinipes*, n=2 for each). This would have reduced pseudoreplication, however, we did account for pseudoreplication in our analyses. Using more colonies is possible for some species that are common and densely populate the area, such as *T. angustula* and *S. depilis*, or are popular with beekeepers such as *Melipona* spp., but is more difficult for *Trigona* colonies that are less common and often built in difficult to access locations such as high on trees or buildings. Despite the low colony number for *T. fuscipennis* and *T. spinipes*, the data for these species still have value in that they are consistent with the overall picture that *Trigona* spp. (n=9 total), are extremely aggressive and the most self-sacrificial in defence of their nests. It is also not uncommon in the study of social insects to take multiple recordings from single colonies. For example, (Couvillion et al. 2008a) made a comparison of 26 stingless bee

species but was only able to locate a single *T. hyalinata* colony, and in a laboratory study of social learning in *Bombus terrestris*, a series of experiments each used worker bees from a single colony (Alem et al. 2016).

A method for quantifying the pain caused by the bites of stingless bees would also be of value. One prediction would be that larger and more aggressive species, or those with sharp mandibles, deliver bites that are more painful. This could be done by submitting blind or naïve observers to bites from various species and correlating the pain experienced with measures of aggression from the flag test bioassays. The use of a small, sensitive strain gauge could also be used to measure biting force (Smith & Palmer 1994, Taylor 2000, Reinhardt & Blickhan 2014).

Our study used a blag flag to elicit defensive responses from bee colonies. The black flag was perhaps most representative of a small to medium sized bird or mammal. However, stingless bees face a wide variety of threats to their nests, such as larger vertebrates and other arthropods including other social insects (section 1.3). While *Trigona* spp. were extremely aggressive towards the flag compared to other species, exposure to other threats may yield a differential pattern in defensive responses. For example, *T. angustula* were only moderately aggressive towards the flag and rarely attack humans, but are known to be extremely aggressive towards *L. limao* robber bees or models carrying their odour (van Zweden et al. 2011). Future studies of defence should take into account the ecological context of the stimulus used to elicit the defensive response.

Chapter 3 – Unique nest entrance structure of *Partamona helleri* stingless bees leads to remarkable 'crash landing' predator avoidance behaviour

3.1 Abstract

The nest entrances of eusocial insects are subject to the conflicting evolutionary pressures of foraging efficiency and defence. Large entrances allow more foragers to pass but require more guards. *Partamona helleri* stingless bees construct a unique entrance funnel, comprising wide outer and narrow inner entrances, which resolves this conflict. We show that this structure has given rise to a remarkable behaviour in returning foragers, which accelerate as they approach the nest and 'crash' head first into the entrance. We compared landing behaviour with two related species with architecturally different entrances that land conventionally using their legs: *Melipona scuttelaris*, whose narrow entrance allows only a single bee to pass and *Scaptotrigona depilis*, which has a wide entrance tube. All three species initially decelerated on their approach to the nest entrance. However, 0.2m from the entrance *P. helleri* began accelerating, whereas the other species continued to decelerate. *Partamona helleri* entered its nest at 1.14 ms^{-1}, double the velocity of the other species. Importantly, *P. helleri* made no fewer landing errors, such as missing the entrance or falling off, than the other species. We then used a bioassay that suggests that this behaviour is a defence against ambush predators at the nest entrance. Finally, we use a scaling argument to show that the crash impact should not cause any damage to a small animal such as a bee, so that no morphological adaptation is required.

3.2 Introduction

Nest building is common across the animal kingdom, and nest location and design are subject to a variety of selective criteria, constraints and trade-offs (Wallace 1867, Barber et al. 2001, Hansell 2007, Casteren et al. 2012, Wenseleers et al. 2013). With few exceptions, eusocial insects build and inhabit nests, which are the central hub for foraging, house the young and reproductive individuals and often contain food stores (Hölldobbler & Wilson 1990, Seeley 2010). The entrance of a social insect nest is of particular importance because it is where the colony meets the wider environment. This interface is subject to conflicting evolutionary pressures, such as foraging efficiency

versus defence. For example, in the stingless bees (Apidae: Meliponini), larger nest entrances allow greater worker traffic but require more guards to defend (Couvillon et al. 2008a).

Some social insects, such as *Apis mellifera*, build their nest in an existing cavity that is chosen according to a set of criteria, but the entrance otherwise receives little modification (Visscher 2007, List et al. 2009, Seeley 2010). However, in the stingless bees, workers build species-specific entrance structures (Michener 2000, Roubik 2006). One stingless bee genus, *Partamona*, has been able to finesse the trade-off between foraging and defence through the invention of a unique funnel-shaped entrance (Camargo & Pedro 2003, Figure 3.1A): The wide outer entrance of the funnel facilitates worker traffic while the narrow inner entrance requires only a few guards to defend (Couvillon et al. 2008a).

Informal observations of *P. helleri* nests in São Paulo State, Brazil indicated that their unique entrances were coupled with a remarkable behaviour not seen in other bees. Rather than slow down as they approached the entrance before landing, returning *P. helleri* workers appeared to accelerate. Then, rather than landing conventionally using their legs as undercarriage, *P. helleri* individuals crashed headfirst into the wall of the outer entrance funnel and fell down towards the inner entrance. Chittka et al. (1997) made a similar observation of *P. pearsoni*, noting that bees did not slow down as they approached the entrance. This behaviour is in stark contrast with other flying insects, which gradually decelerate to near zero velocity, hover and extend their legs in a controlled fashion (Srinivasan et al. 2000, Evangelista et al. 2010).

Why has this unusual landing behaviour evolved? It is unlikely to increase foraging efficiency, because the time saved over the final nest approach is likely negligible compared to the whole foraging trip. Another possibility is that it is a predator avoidance mechanism. The nest entrance is a focal point for worker activity, and ambush predation at the entrance can lead to significant losses in the colony workforce (Mackay 1982, Schatz & Wcislo 1999). We observed two such ambush predator groups on *P. helleri* nests at our study site, Salticidae (jumping spiders, Figure 3.1D, E) and

Reduviidae (assassin bugs), and we have further observed video footage of *Tropidurus* spp. lizards predating on *P. helleri* workers at the entrance (Zacaris 2015). Bees are probably at their most vulnerable to these entrance predators when landing, before they have entered the safety of the nest. However, faster moving prey should be more difficult to capture (van Damme & van Dooren 1999). Therefore, we hypothesize that the acceleratory behaviour of *P. helleri* is a mechanism for returning foragers to reduce predation.

This study investigates the crash-landing entrance behaviour of *P. helleri* in four parts. First, we quantify the natural history of the nest approach and landing behaviour of *P. helleri* bees and compare this with two other stingless bee species with contrasting entrance structures: *Melipona scutellaris* which has a narrow entrance through which only one or two bees may pass at a time (Figure 3.1B), and *Scaptotrigona depilis* which has a wide, tubular entrance suitable for high worker traffic, the ancestral state of *P. helleri* (Figure 3.1C). Second, we investigate whether this behaviour results in a greater amount of errors when entering the nest. Third, we use a bioassay to test the hypothesis that crash-landing has evolved as a predator avoidance mechanism. Finally, we address the biomechanics underlying the crash-landing behaviour.

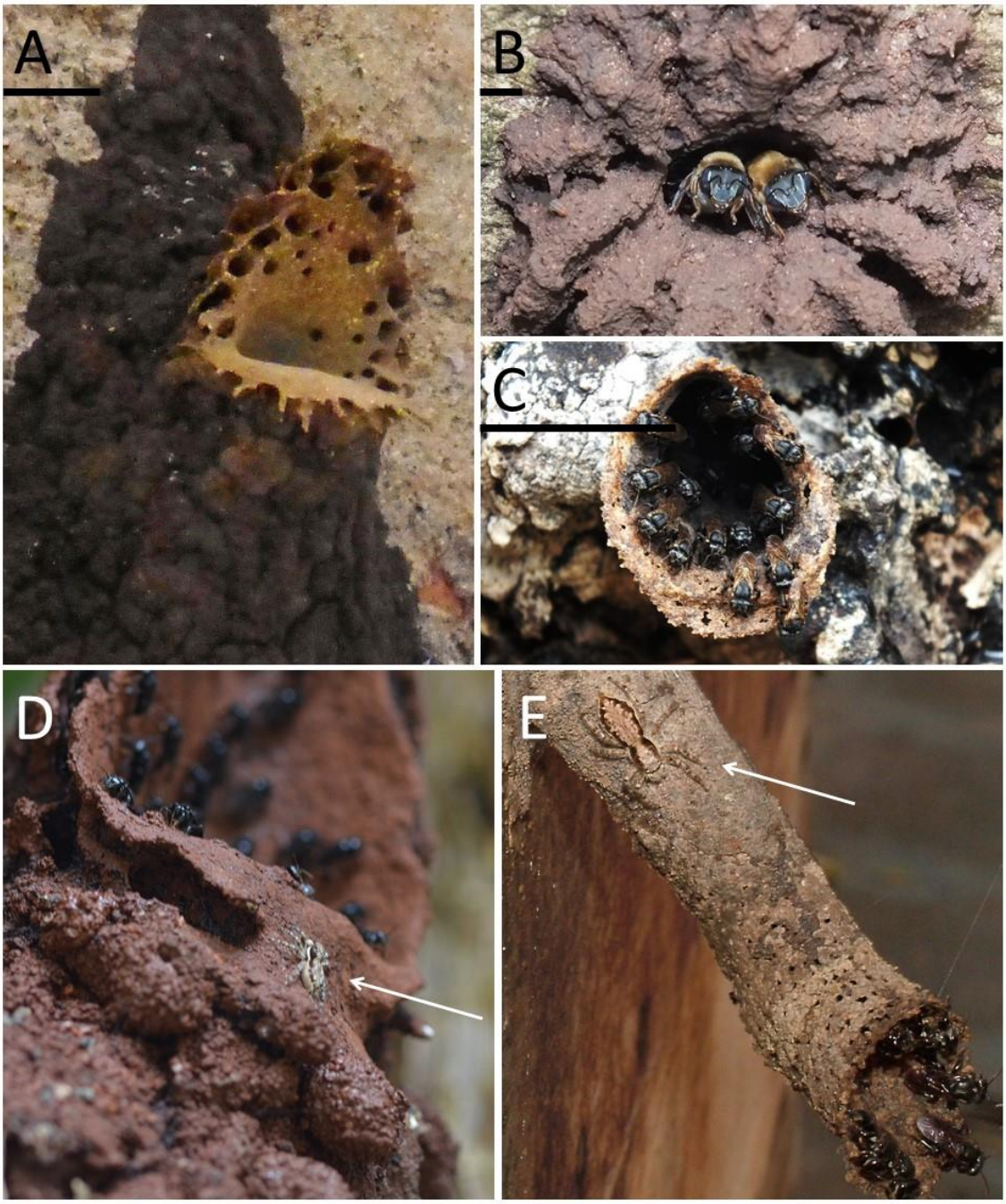

Figure 3.1: Nest entrances of the three stingless bee study species from colonies in São Paulo State, Brazil. **A** outer funnel entrance of *Partamona helleri* built from soil and resin, **B** narrow entrance hole of *Melipona scutellaris* built from soil and resin, **C** broad entrance tube of *Scaptotrigona depilis* built of wax and resin, São Paulo State, Brazil. All scale bars approximately 20mm. Workers may face ambush predation at the nest entrance, for example by Salticidae jumping spiders **D** and **E**.

3.3 Methods

Site description and study species

The study was carried out on the campus of the Luiz de Queiroz College of Agriculture (Escola Superior de Agricultura Luiz de Queiroz, ESALQ), Piracicaba, São Paulo State, Brazil, from 2015-2017 during February and March each year. Data were collected when bees were foraging from 06:00-11:00 at temperatures of 20-30C.

We studied colonies of *Partamona helleri*, *Melipona scutellaris* and *Scaptotrigona depilis*. All *M. scutellaris* and *S. depilis* colonies were housed in hives while *P. helleri* colonies were in both hives and wild nests. All colonies had constructed their unique nest entrance structures (Figure 3.1).

Speed and acceleration of approaching bees

A Panasonic DMC-TZ30 camera was set up perpendicular to the approach flights made by bees as they returned to a nest entrance. A white background, either the wall of a building or piece of plywood, acted as a backdrop against which the dark-coloured bees could be easily seen on the video. The background was marked at 0.1m intervals beginning 1.5-1.4m from the nest entrance, enabling us measure the time taken to travel measured distances from the video, and thereby calculate the speed and acceleration over each interval. We studied at this scale because preliminary observations had shown that most bees were aligned with the entrance and flying directly towards it at this distance. We used the high-speed video setting to record at 220 frames-per-second, enabling us to play back the recordings in slow-motion and more accurately measure flight speed. The camera was approximately 5.0m from the flight path and the white backdrop was approximately 0.2m further back creating a small parallax error that we corrected for in our analysis. We studied three nests of each species and recorded 89 individuals of *P. helleri*, 65 of *M. scutellaris* and 28 of *S. depilis*.

Could speed changes be due to elevation changes?

We investigated whether any changes in flight velocity could be attributed to changes in activity on the part of the bee, or due to elevational changes, in which kinetic energy is converted into gravitational potential energy, or *vice versa*. We used the same camera

settings as when filming approach velocity, but only analysed distances from the entrance at which velocity changes were occurring. We measured the elevation changes of bees relative to their change in speed for 30 bees of each species.

Landing errors of bees approaching the entrance

Some returning bees are unsuccessful at entering the nest. In *P. helleri* some bees hit and bounce out of the entrance or collide with the rim of the funnel. In *M. scuttelaris* collisions occur with departing nestmates and in the crowded *S. depilis* entrance some bees fall off the rim. To quantify these errors, we set up the camera 4.0m from the entrance at a horizontal angle of 45° and zoomed in to get a clear view. We recorded 295 bees for *P. helleri*, 300 for *S. depilis* and 200 for *M. scutellaris* (6 nests per species) and then analysed the videos to determine the proportion of bees that successfully entered their nest versus those that made landing errors.

Predation bioassay

We tested the hypothesis that *P. helleri* accelerate on approaching the nest entrance to avoid ambush predation. We collected and freeze-killed jumping spiders (Salticidae) occurring naturally within three metres of the *P. helleri* study colonies (Figure 3.1B). Spiders were not identified to species but varied in colour and in length from 5-10 mm excluding appendages. We applied a 'predator treatment' to colonies by placing four spiders on the outer entrance of a *P. helleri* nest and compared the speed of bees approaching these nests with control nests. We again focussed on the distance at which we had identified acceleration changes, and measured speed across small, 25mm, intervals filming at 220 FPS. We studied three nests and rotated the predator and control treatments around each. In total we recorded 90 approaching bees under the predator treatment and 180 for controls (n = 3 nests, evenly divided per nest). We further examined whether any speed changes resulted in increased errors as above.

Statistical analysis

We had no *a priori* knowledge of the scale at which acceleration changes were likely to occur. Therefore, we first visually inspected the data for bees approaching the entrance to identify likely points of speed change. We then compared acceleration among species

before and after these points using a mixed-effects model, fitting colony as a random effect. To analyse the error rate of bees among species (proportion of successful landings), we used a mixed-effects model with a binomial error structure fitting species as a fixed-effect and colony as a random effect. For the predation bioassay, we compared bee velocity before and after identified points of velocity change using a mixed-effects model, fitting treatment (spiders versus control) as a fixed-effect and colony as a random effect, and examined error rates using a mixed effects model with a binomial error structure. Reported test statistics are comparisons to the null model using ANOVA. All statistical analyses were performed using R 3.1.1 (R Core Team 2014) and the lme4 package (Bates et al. 2015).

3.4 Results

Speed and acceleration of approaching bees

Partamona helleri and *Melipona scutellaris* both initially travelled at a similar velocity of approximately 2.0ms^{-1} (Figure 3.2A). *Scaptotrigona depilis* was initially faster at 2.6ms^{-1}. However, samples sizes were low for *S. depilis* for distances >1.0m as most individuals were not yet aligned to their entrances, which led to wider standard errors (Figure 3.2A). All *S. depilis* individuals were aligned by 0.7m, at which point it was the slowest of the three species. Approach velocities reduced for all three species as they neared the entrance. However, at 0.2m from the entrance, the velocity of *P. helleri* increased while it continued to decrease in the two other species. *Partamona helleri* workers collided with the entrance at 1.14 ms^{-1}, double that of the controlled landing in the other two species.

To investigate this result further, we analyzed the acceleration of bees near the entrance, 0.2-0.0m, compared with greater distances 1.4-0.2m. We found significant differences in acceleration among species, and for greater versus less than 0.2m from the entrance (mixed-effects model, $P < 0.001$, $\chi^2 = 183.02$, DF = 5, Figure 3.2B). *Post hoc* tests showed no significant differences in acceleration among species at 1.4-0.2m ($P>0.05$ in all cases). However, at 0.2-0.0m, the acceleration of *P. helleri* was significantly greater than the other species ($P<0.001$ in all cases) and this was the only instance in which acceleration was positive. That is, *P. helleri* was accelerating within the final 0.2m of

approach while both *S. depilis* and *M. scutellaris* continued to decelerate. Crucially, the acceleration of *P. helleri* over 0.2-0.0m was significantly greater than over 1.4-0.2m ($P<0.001$). This shows that as *P. helleri* approached the nest entrance, its acceleration switched from negative to positive. The other two species did not express this switch. *P. helleri* concluded its entry by crashing, head-first, into the entrance.

Elevation changes

Over the final 0.2m of approach to the entrance, *P. helleri* first increased, then decreased its height, such that its total height change was only -1.83mm (Figure 3.2C). We calculated the changes in kinetic energy (KE) and gravitational potential energy (GPE) experienced by *P. helleri* over this period. *Partamona helleri* individuals have a wet mass (M) of 0.0108 ± 0.00282g (Table 3.1), their velocity (V) changed from 0.94 ms^{-1} at 0.2m from the entrance to 1.14ms^{-1} at 0.0m (Figure 3.2A), their height (H) changed by -1.83mm and they were under the influence of gravitational acceleration (G) of 9.807ms^{-2}. Using the formula $KE = MV^2 / 2$, this equates to an increase in kinetic energy of 2.2×10^{-6}J. Using the formula GPE = MGH, *P. helleri* bees lost only 1.94×10^{-7}J of GPE. Therefore, the loss in GPE accounts for only 8.8% of the gain in KE, which suggests that the acceleration of *P. helleri* in the final 0.2m of approach was mainly due to energy provided through the wings and flight muscles.

We observed a contrasting pattern of height change in *M. scutellaris,* which gained 0.0345m in height over the final 0.2m. Using the formula $MV^2 / 2 = MGH$, this height gain would result in a velocity loss of 0.82 ms^{-1} (the actual velocity decrease of *M. scutellaris* in the final 0.2 m was 0.43 ms^{-1}, Figure 3.2A). Thus the height gain of *M. scutellaris* was more than sufficient to reduce its velocity to 0 ms^{-1}, and it must therefore generate additional thrust with its wings. Hence, while the approach angle of *P. helleri* probably does not significantly increase its velocity, neither does it reduce it as in *M. scutellaris*.

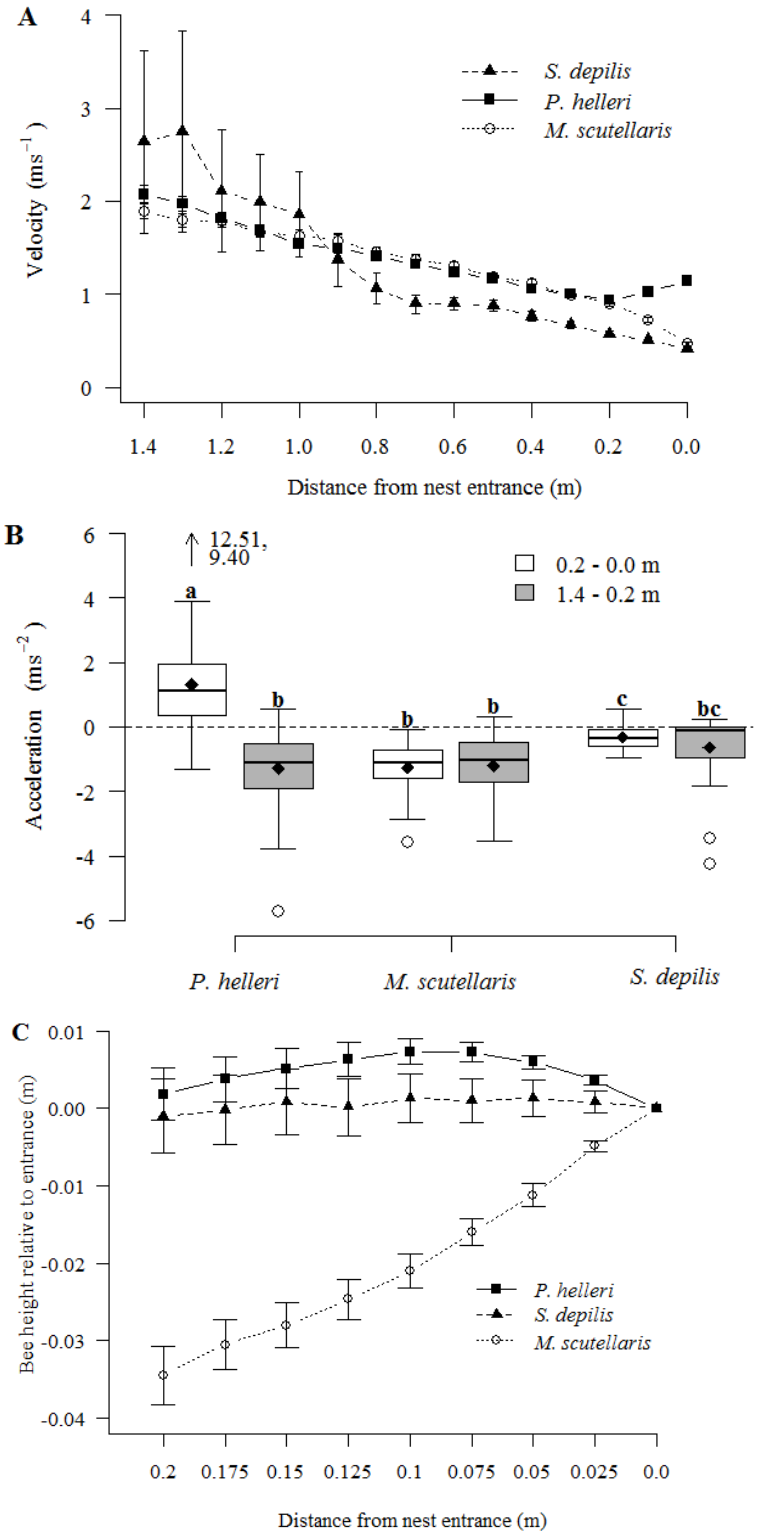

Figure 3.2: **A** mean velocity of three stingless bee species as they approach their nest entrances, error bars ± 1 standard error. **B** acceleration before and after 0.2m from the entrance. Letters show significant differences. Bars indicate 1.5× IQR, black diamonds show means, white circles and arrows show outliers, dashed line shows the boundary between acceleration and deceleration. **C** height change. The height of 0.00m at 0.0m horizontal distance from the entrance represents the point of entry into the nest.

Table 3.1: Descriptive measures of three stingless bees. Both bee heads and nest entrances are approximately elliptical. Therefore, areas were calculated using the same formula, $\pi \times (\text{height} / 2) \times (\text{width} / 2)$, n=10 bees per species (outer entrance in the case of *P. helleri*). Outward velocity measured at 1.5-1.6m from entrance as in Methods for approaching bees, n=30 per species. All values are means ± 1 standard deviation.

Species	Wet mass (g)	Entrance area : head area	Outward velocity (ms^{-1})
Partamona helleri	0.011 ± 0.003	725.4 ± 182.3	4.57 ± 0.91
Melipona scutellaris	0.082 ± 0.006	9.5 ± 0.5	4.47 ± 1.00
Scaptotrigona depilis	0.015 ± 0.001	174.4 ± 106.0	3.57 ± 0.84

Landing errors of bees approaching the entrance

The vast majority of individuals from all three species successfully landed or crash-landed at their nest entrance: 98.0%, 96.5% and 94.0% for *P. helleri*, *M. scutellaris* and *S. depilis* respectively (n = 295, 200 and 300 respectively). In the fast moving *P. helleri*, six bees hit the back of the outer entrance and bounced out of the entrance funnel rather than falling down and toward the inner entrance, while one bee hit the lip of the entrance, bouncing outwards. In the slower moving *M. scutellaris*, the landing of seven individuals was disrupted by traffic; either a nestmate attempting to enter simultaneously or colliding with an outward-bound bee. In *S. depilis*, the high density of guards posted around the entrance (Figure 3.1C) hindered returning bees, such that there was little available space for them to land, resulting in 18 bees tumbling down from the entrance. After missing, bees fell downwards a few centimetres to a metre in the faster moving *P. helleri*, but not as far as the ground, and attempted to enter their nest again seconds later. Bee species did not differ significantly in the proportion of individuals successfully entering the nest (mixed-effects model, P = 0.2311, $\chi^2 = 2.93$, DF = 2).

Predation bioassay

The average speed of *P. helleri* bees did not differ between spider and control treatments at 0.2-0.4m from the nest (mixed-effects model, P = 0.219, $\chi^2 = 1.508$, DF = 1). However, in the final 0.2m, bees which approached nests with experimentally added

spiders (predator treatment) had significantly greater velocity than those approaching control nests, 1.21 ms^{-1} versus 1.11 ms^{-1}, a 9% increase (mixed-effects model, P = 0.004, χ^2 = 8.202, DF = 1, Figure 3.3). This did not result in a greater number of errors, as 98.0 vs 98.8% of control versus predator treatment bees successfully entered their nests (mixed-effects model P = 0.372, χ^2 = 0.798, DF = 1). Informal observations indicated than approaching bees did not deviate from their course in the presence of spiders, but entered the nest in their usual manner.

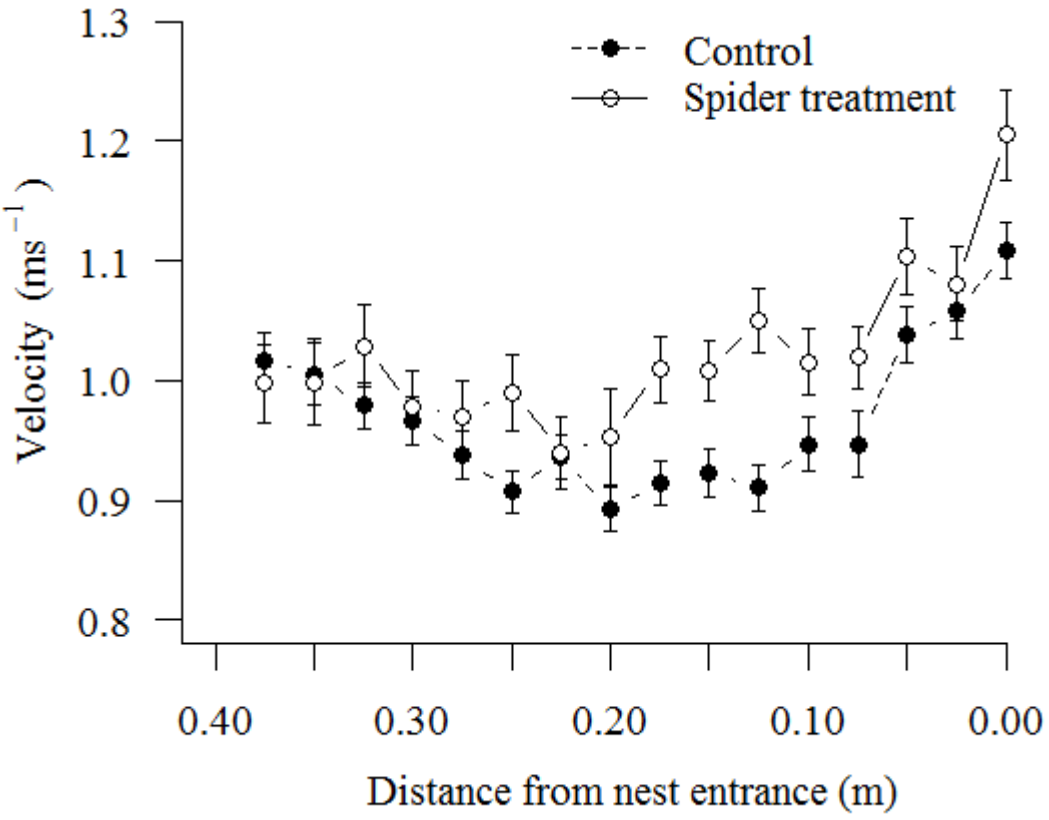

Figure 3.3: Velocity of *Partamona helleri* stingless bee foragers approaching nests with and without the presence of predators, dead jumping spiders (Salticidae), placed on the outer edge of the entrance funnel. Error bars ±1 standard error.

3.5 Discussion

Our results show that *Partamona helleri* workers accelerate in the final 0.2m of their approach to the nest entrance. This was in contrast to *Melipona scutellaris* and *Scaptotrigona depilis*, both of which reduced their velocity throughout the final 1.4m of their approach and landed conventionally using their legs. At the point of entry, the velocity of *P. helleri* was double that of the other species. This shows that *P. helleri* have a distinct behavioural sequence in approaching the entrance. To an observer, returning *P. helleri* workers appear to initially slow down on their approach and 'aim' themselves at the funnel entrance. They then accelerate so that they enter the outer entrance and crash into its back wall, then tumble down into the inner entrance. This is probably aided by the large area of the funnel entrance (Couvillon et al. 2008a), which is also a visually contrasting target (Chittka et al. 1997). The evolution of the funnel entrance seems to have resulted in the evolution of a novel landing behaviour consisting of both the acceleratory behaviour and the crash.

Despite accelerating, the crash-landing velocity of *P. helleri* was relatively low compared to what a bee in free flight can achieve. Bees crash-landed at 1.14 ms^{-1}, while the velocity of outward flying bees 1.5m from the entrance was 4.57 ± 0.91 ms^{-1}, n=30, Table 3.1). This seems a reasonable estimate of the free flight speed of *P. helleri*, given that the larger *Apis mellifera* flies at approximately 7 ms^{-1} (Wenner 1963). Combined with the requirement for *P. helleri* to initially slow down and align itself with the entrance, the speed difference between arriving and departing bees suggests that there are constraints on the maximum velocity a crash-landing bee can attain. Alternatively, *P. helleri* may be capable of greater acceleration but the benefits of doing so are small.

There was surprisingly little variation in the velocity of bees in all three species, particularly when they neared the nest entrance (Figure 3.2A). In the case of *P. helleri* there are likely biological limitations on the maximum velocity at which it can accurately resolve the image of its entrance (when it slows to its minimum speed at 0.2m), and then the maximum acceleration that is possible to attain. These limitations are likely very similar among workers in a population. For example, there is likely more variation in size and speed between two randomly selected human females than between

two randomly selected *P. helleri* workers. In the case of *S. depilis* and *M. scutellaris*, both species were tending towards a speed of 0 ms^{-1}, which would partly explain their lack of variation. The greatest variation was seen among *S. depilis* bees >0.7m from the entrance. This was because not all bees observed had aligned themselves with the entrance until this point, and sample size was therefore lower (section 3.4). The small standard errors of *P. helleri* bees may also be attributed to its large sample size (n=95).

Bees approaching the nest entrance do not appear to adjust their posture in preparation for a crash. The behavioural sequence can be seen in Figure 3.4. Bees fly in a straight line in a regular flight posture (Figure 3.4A), and do not appear to adjust their posture even just before making contact with the entrance (Figure 3.4B). On making contact with the wall of the entrance, the body of the bee appears to compact (Figure 3.4C), before it falls straight down towards the inner entrance (Figure 3.4D). Video footage using a dedicated high-speed camera would better elucidate this behavioural sequence.

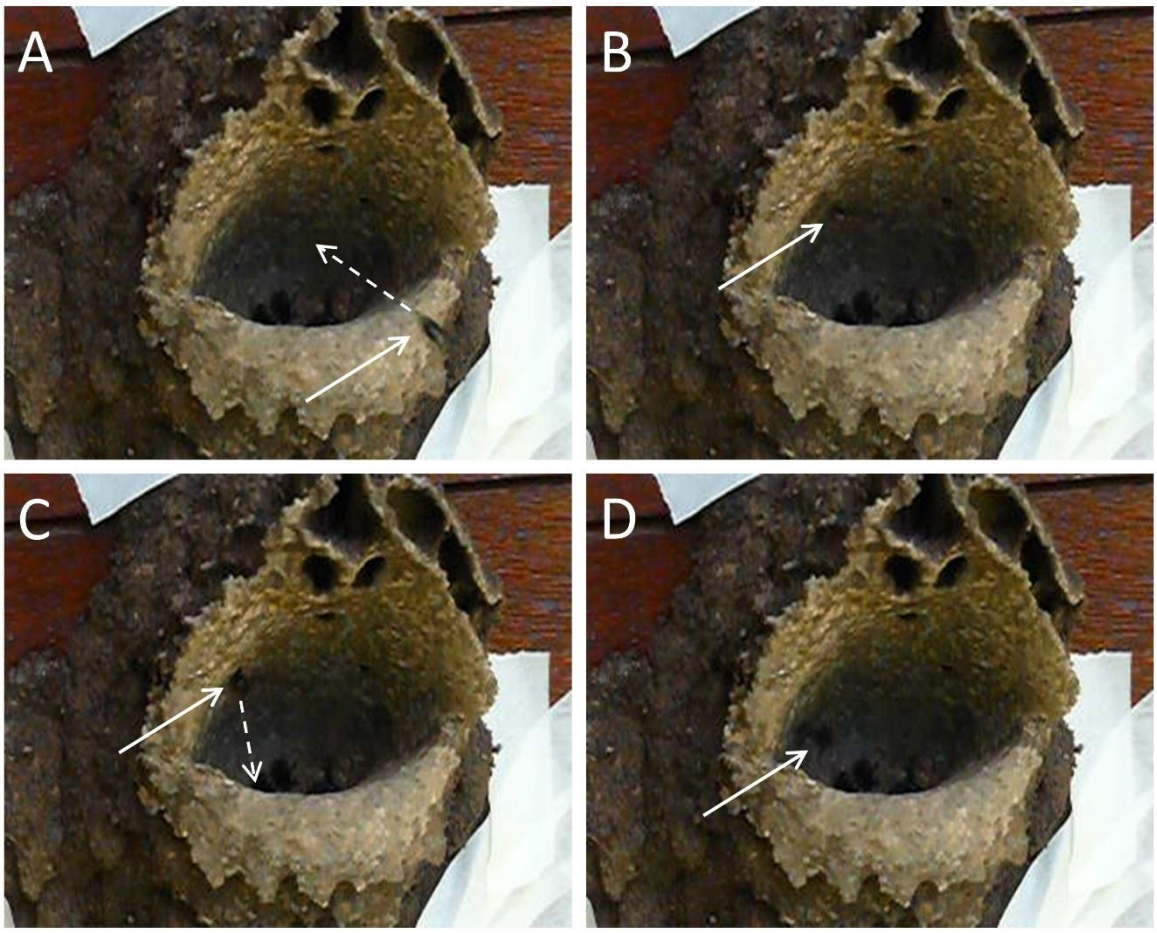

Figure 3.4: Sequence of a *Partamona helleri* worker approaching its nest entrance and crash landing. Solid arrows show the location of the bee. Dashed arrows show the direction of travel.

Despite landing at double the velocity of the other two species, *P. helleri* made fewer landing errors than the other two study species, although not significantly so. *Melipona scutellaris* has only a small area to aim at, while *S. depilis* has a crowded entrance with many guards. By contrast, *P. helleri* has a large entrance area–head cross-sectional area ratio (Table 3.1), which coupled with a bold visual target at which to aim (Chittka et al. 1997), likely facilitate its ability to enter its nest at higher velocity. Because *P. helleri* guards are stationed at the lower, inner entrance, the chance of collisions with other bees is also low. The costs of making a mistake are probably not great in terms of time lost, as it would take only a few seconds to reattempt entry. The costs of errors would be greater when predation pressure at the entrance is high, a greater penalty for missing.

Like other animals, social insects are known to trade-off speed and accuracy in other aspects of their biology, such as foraging decisions (Chittka et al. 2003, Burns & Dyer 2008, Ings & Chittka 2008, Chittka et al. 2009). However, it is not clear whether the lack of difference in error rate of bees entering their nest constitutes a lack of difference in accuracy, as innacurate bees are less likely to be punished when the entrance is large as in the case of *P. helleri*. Studies comparing the velocity and error rates of crashing landing workers among *Partamona* nests of varying entrance sizes would therefore be of interest, as would experimental manipulations of the entrance size.

The presence of Salticidae spiders caused *P. helleri* to speed up to a greater degree than when spiders were absent. This suggests that bees were able to detect the spiders and exhibited an adaptive response by entering the nest at greater velocity. Our results parallel those of Tan et al. (2007), who found that *Apis cerana* bees reduced predation from bee-hawking by *Vespa* wasps by speeding up their nest approach. We do not know whether *P. helleri* recognized the experimental spiders as dangerous predators, or simply that something unusual was present at the nest entrance. Neither do we know the level of natural predation pressure which nests were under at the time of the experiment, other than that predators were present on and near nests.

Defence in social insects is usually viewed in the context of defending the whole nest (Ono et al. 1995, Couvillon et al. 2008a, van Zweden et al. 2011), sometimes at the cost of the lives of defenders (Shorter & Ruepell 2012). However, individual level defences are also important, as each forager lost to predation reduces the ability of the colony to gather food, so that both the colony and the individual lose fitness. While *P. helleri* is relatively aggressive in the collective defence of its nest (Shackleton et al. 2015), the acceleration behaviour is an example of predator evasion at the individual level, combined with the colony-level adaptation of the funnel entrance. It would be interesting to study *Partamona* species with funnel entrances alongside their congeners with tube entrances, the ancestral state (Camargo & Pedro 2003), to compare landing behaviour, errors and predation.

The funnel entrance of *P. helleri* probably did not evolve in response to individual-level predation. Rather, foraging constraints and nest-level predation are likely the greater selective pressures (Couvillon et al. 2008a) and the structure then led to the evolution of the acceleratory behaviour. Exaptations (Gould & Vrba 1982) occur at all levels of organismal organization, from the molecular to behavioural (Yosef & Whitman 1992, Armbruster et al. 1997, Ketterson & Nolan Jr. 1999). Our results and behavioural observations show how exaptations can occur in the extended phenotype (Dawkins 1982) of an organism, its nest.

The purpose of crashing head-first in to the entrance is more difficult to discern. Travelling at a greater speed may make a conventional landing impossible, and crashing may be a side effect with no great drawback. Larger animals that experience head impacts such as woodpeckers (Picidae) and head-butting goats (Bovidae) have evolved morphological adaptations to protect the brain from damage (Gibson 2006, Farke 2008, Figure 3.5A). Similarly, humans who engage in activity involving head collisions, such as American football, wear protective headgear (Figure 3.5B). However, *Partamona helleri* workers, which repeatedly crash land into the "brick wall" of their nest entrance, have no apparent external morphological specializations, when compared to the two other stingless bees studied (Figure 3.5C-F), although we cannot rule out the presence of internal adaptations. The low mass of *P. helleri* combined with scaling laws mean that the forces it experiences during a crash are probably not great (section 3.5.1). This suggests that despite the drastic evolutionary change in the landing behaviour of *P. helleri*, no corresponding morphological adaptation is required.

Biomechanics of Partamona helleri crash-landing behaviour

Collision damage, especially to the head, can have serious fitness consequences, even death. Humans walk at a similar speed to a crash landing *P. helleri* worker (1.14 ms^{-1}). It is common knowledge that a head impact at walking speed can hurt and cause harm to a human, and that humans who are likely to experience impacts at high speeds often wear a crash helmet (Figure 3.5B). How can *P. helleri* bees routinely crash without apparent protection? Through scaling effects, the physical challenges faced by organisms vary with body size (Haldane 1926). The damage caused by an impact depends on the kinetic energy (KE) of the object, which for a given velocity is proportional to its mass, and the area over which the impact occurs. Mass scales as the cube of an objects length, whereas area scales as length squared. Small objects have a lower mass per unit area than large objects. Therefore, the impacts bees experience are less damaging than to humans.

Consider the forces that act upon a crash-landing *P. helleri* worker and a human travelling at 1.14 ms^{-1}. The mass of a *P. helleri* worker (10.8 mg) is approximately 6 million times less than an adult human (62 kg). From this, we can calculate the KE (0.5 × mass × $velocity^2$) for each (7.0E-6 versus 40.3 J respectively). The cross sectional area head of a bee is 4.1E-6 m^{-2} versus 2.25E-2 m^2 for a human. The toughness of insect cuticle and bone, measured as the work of fracture in joules per metre squared, is comparable (1500 versus 1700 Jm^{-2}, Vogel 2003). From these figures, we can calculate the impact experienced during a crash. Importantly, the impact experienced a *P. helleri* worker at crash-landing speed (1.7 Jm^{-2}) is far lower than its work of fracture (1500 Jm^{-2}), and 1000 times less than that of a human (1791 Jm^{-2}), which experiences an impact close to its work of fracture (1700 Jm^{-2}). Thus, while the evolution of acceleratory and crash-landing behaviours in *P. helleri* was likely enabled by the pre-existing funnel entrance, no morphological adaptation is required.

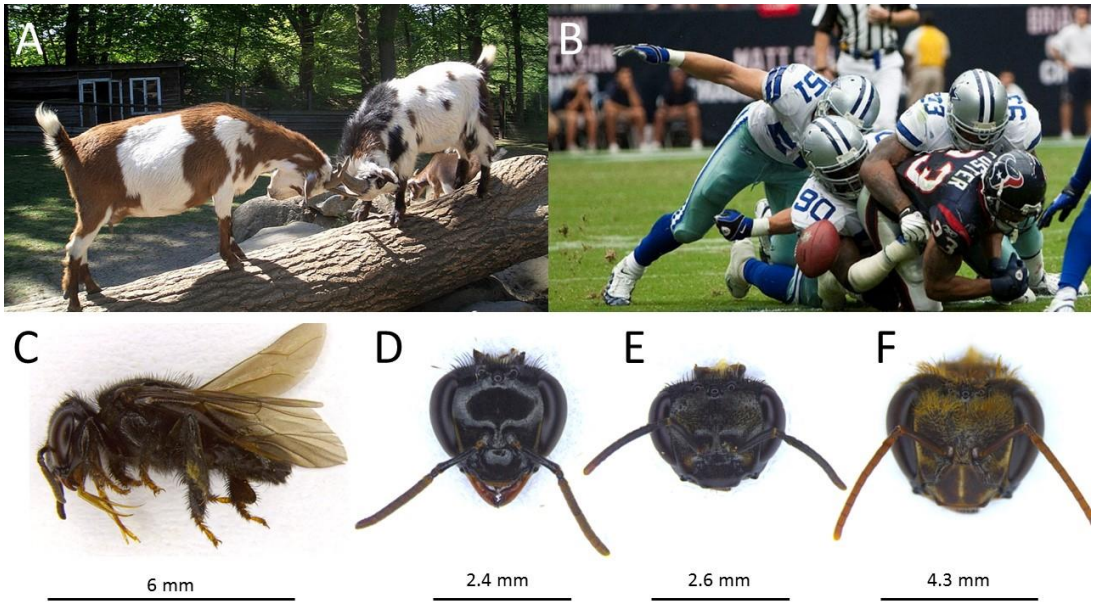

Figure 3.5: Goats **A**, and humans playing contact sport **B**, commonly have a form of 'crash helmet' to reduce impact damage. The crash landing workers of *Partamona helleri* stingless bees **C**, **D**, do not appear to have any external morphological features that would reduce impact damage over related species that do not crash land, *Scaptotrigona depilis* **E**, and *Melipona scutellaris* **F**, probably by virtue of their small size.

Chapter 4 – Spatial organisation enhances collective vigilance in the hovering guards of *Tetragonisca angustula* stingless bees

4.1 Abstract

One benefit of group living is vigilance against predators. Previous studies have investigated the group size effect, where individual vigilance decreases as group size increases but overall predator detection by the group may increase. However, there has been comparatively little research on whether the positioning of individuals can improve the collective vigilance of the group. We studied the coordination of vigilance and its effect on predator detection in the eusocial bee *Tetragonisca angustula*. Nests are defended by hovering guards that detect and intercept intruders before they reach the nest entrance, in addition to those that stand upon it. Hovering guards varied in their position in all three dimensions, but showed greatest variation along an axis spanning the left and right of the entrance. Guards then faced inwards, forming a corridor, through which incoming bees must pass. We show that hovering guards are positioned non-randomly along the left-right axis, with a strong tendency for equal numbers on both sides of the entrance. This organisation increases the collective vigilance of the guard group, as in groups of two the collective field of view is greater when guards are positioned on both sides versus only one side. Last, we use a bioassay to show that when guards are on both sides of the entrance, their ability to detect intruders before they reach the entrance is increased. Overall, our results provide strong evidence that vigilance is positionally coordinated, and that this improves nest defence. Whereas other group-living animals are often selfish in their individual vigilance behaviours, and face competing time constraints such as foraging, social insect guards have largely sacrificed their direct fitness and do not need to forage, which has likely facilitated the evolution of coordinated vigilance, as documented here in *T. angustula*.

4.2 Introduction

Vigilance against predators is one potential benefit of group living, as it can increase predator detection and individual survival (Pulliam 1973, Krebs & Davies 1993, Cresswell 1994, Beauchamp 2017). An increase in group size also leads to a reduction in the time spent vigilant of individual group members (Bertram 1980, Elgar & Caterall

1981, Lima 1995). The group size effect is commonly explained by either the increased detection of predators by the group, known as the many-eyes hypothesis (Lima 1995, Fairbanks & Dobson 2007), or the dilution effect whereby each individual is at lower risk of being targeted by a predator (Hamilton 1971, Dehn 1990, Roberts 1996). By spending less time vigilant, individuals can dedicate more time to foraging or other activities that enhance fitness (Elgar & Caterall 1981). For example, Lima (1995) found that dark-eyed juncos, *Junco hyemalis*, consumed food items over 50% faster as group size increased from one to six.

The collective vigilance of a group would be increased if group members also coordinated their vigilance efforts, such as by looking in different directions. Alternatively, coordinated vigilance may be organised so that some individuals focus on vigilance allowing others to focus on foraging. Although models predict benefits of coordination to collective vigilance (Bednekoff & Lima 1998, Ferrière et al. 1999), such behaviour is rarely observed in nature (Ward 1985, Pays et al. 2007). This may be because individuals are selfish (Hamilton 1971), or because the need to monitor the vigilance status of neighbours is itself costly, and provides only marginal benefits over uncoordinated vigilance (Ward 1985, Rodríguez-Gironés & Vasquez 2002). Where coordinated vigilance has been observed, it usually involves a sentinel system of only one or two vigilant individuals (meerkats; Clutton-Brock et al. 1999, cranes; Ge et al. 2011, rabbitfish; Brandl & Bellwood 2015). However, how vigilant individuals position themselves relative to each other, and how this affects collective vigilance has received less attention than the effect of group size.

Vigilance in social insects differs from most vertebrate examples in that rather than fleeing from predators, vigilance may improve the defence of a fixed location, the nest. The nest contains reproductive individuals, offspring (brood), and food stores, meaning that nest defence provides large fitness benefits. Early detection of predators is often important for social insects because the first predators to arrive are often scouts of other social insect colonies that can recruit nestmates for a mass attack (Blum et al. 1970, Michener 1974, Ono et al. 1995). Detecting and disabling these scouts is, therefore, critical for colony survival. The second important distinction is that social insects often

possess dedicated defenders (guards), which sometimes have morphological specialisations and are not constrained by the need to forage or reproduce. Rather, the trade-off should exist at the colony level through division of labour, in terms of the allocation workers to different tasks.

The stingless bee *Tetragonisca angustula* (Apidae: Meliponini) presents an excellent opportunity to study the group-level coordination of vigilance. In addition to guards that stand at the nest entrance, which is normal in social insects, *T. angustula* colonies also have hovering guards that position themselves near the entrance in flight (Grüter et al. 2011). To date, hovering guards are only known in *T. angustula* and the closely related *T. fiebrigi*. Hovering guards are normally positioned to the left and right of the entrance and face inwards to form a corridor through which most bees entering the nest must pass (Figure 4.1A, Wittman 1985). Guards inspect incomers, intercept non-nestmates, and wrestle them to the ground (Wittman et al. 1985). In agreement with studies of vigilance in vertebrates, larger groups of hovering guards are better at detecting intruders (van Zweden et al. 2011). Furthermore, *T. angustula* guards are morphologically specialised, being the first described and most prominent example of a soldier caste within the bees (Grüter et al. 2012, Grüter et al. 2017a). The main natural enemy of *T. angustula* is the obligate robber bee *Lestrimelitta limao* (Figure 4.1B), which probably drove the evolution of the soldier caste (Grüter et al. 2017a) and whose local density influences colony investment in defence (Segers et al. 2016).

We investigated how *T. angustula* hovering guards are positioned relative to each other and how this affects vigilance and predator detection. Guard position and orientation was first quantified in three dimensions. We looked for where variation in guard position was most apparent (i.e. along which axes), and then concentrated our efforts in determining how guards were distributed along these axes. We predict that guards should coordinate their positioning, such that they are evenly distributed so as to cover a wider area. We then compared vigilance of guard groups that were evenly distributed versus those that were skewed in one direction. Finally, we investigated the effect of guard distribution on the ability to detect predators using a bioassay to simulate an attack.

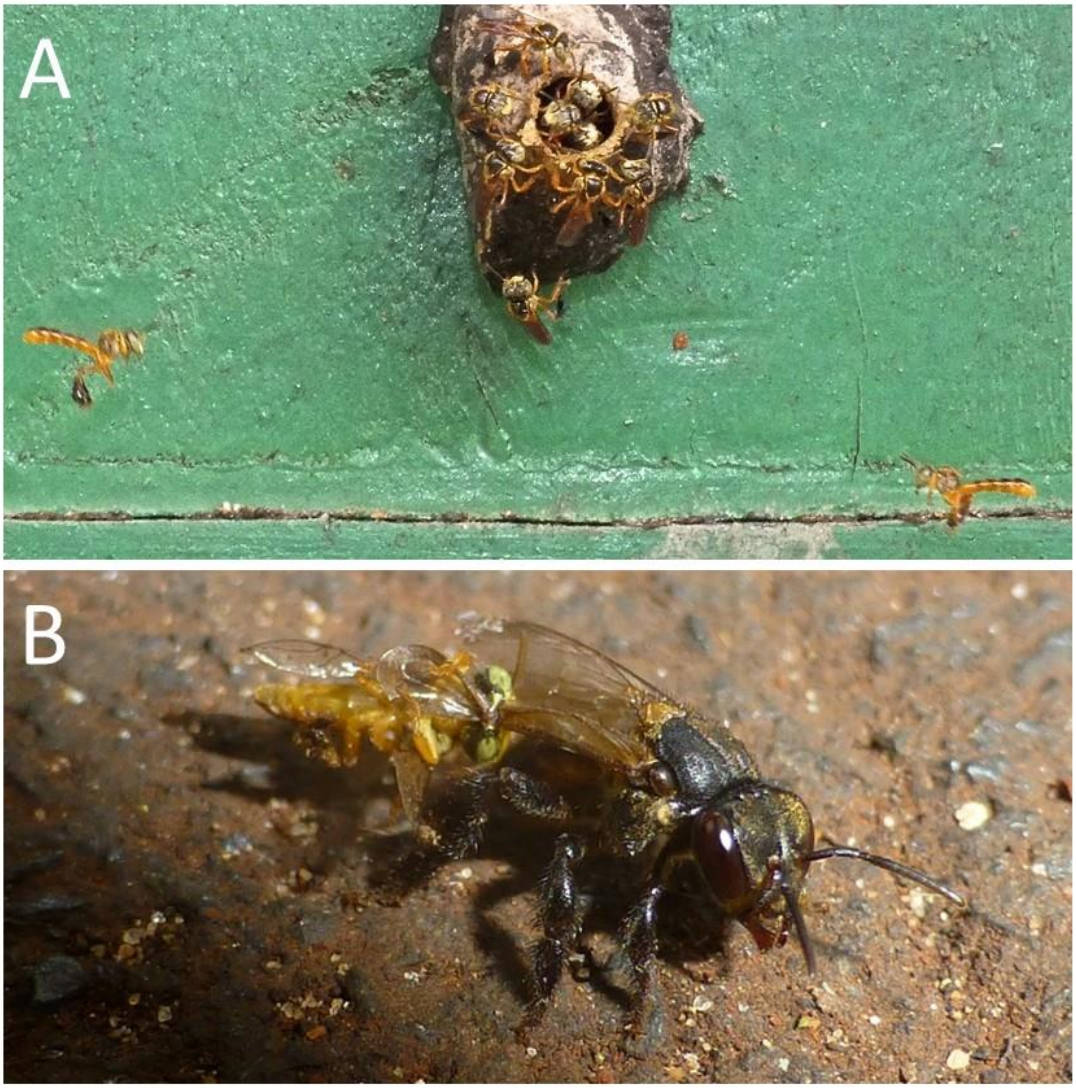

Figure 4.1: **A** Hovering guards of the stingless bee *Tetragonisca angustula* at a nest entrance in São Paulo State, Brazil. Hovering guards are positioned on either side of the entrance tube to form a corridor through which incoming bees must pass. **B** *T. angustula* guard (left) fighting with *Lestrimelitta limao* robber bee (right).

4.3 Methods

Study site and colonies

The study was carried out at the Department of Entomology and Acarology at the University of São Paulo, Piracicaba, Brazil. Data were collected in March 2017 from 08:30-16:30 in good weather conditions when colonies were active. We studied 15 colonies of *Tetragonisca angustula* stingless bees (Meliponini: Apidae), including four wild colonies and 11 in hives. This species is considered mildly aggressive amongst the stingless bees (Shackleton et al. 2015), but is especially aggressive towards the robber bee *Lestrimelitta limao* (Sakagami et al. 1993, Grüter et al. 2012). All colonies had built their characteristic entrance tube and had standing guards positioned at the nest entrance (Figure 4.1A). At the end of each day of data collection, we removed the hovering guards from each colony entrance to minimise pseudoreplication that might arise from gathering data from the same individuals across days.

Three dimensional positioning and facing of hovering guards

Hovering guards can be distributed in three dimensions. We thus defined three axes relative to the nest entrance: a left–right axis, behind–in front of the entrance, and above–below the entrance (Figure 4.2). The position of hovering guards along these three axes was quantified using video footage. Videos taken in a top down view could measure the left–right and behind–in front of axes, and videos taken head on to the entrance could measure the left–right and above–below axes. For each video of 50 seconds we counted the number of guards present and extracted five still images at random intervals. We imported the images into ImageJ (Schneider et al. 2012), used the angle tool to measure the angle of each bee relative to the entrance, and calculated the two-dimensional coordinates in Cartesian space. From this, we were able to plot the distribution of hovering guards and compare their distribution along the three axes. We compared the distribution along the three axes, left versus right, above versus below, and behind versus in front, using chi-squared tests.

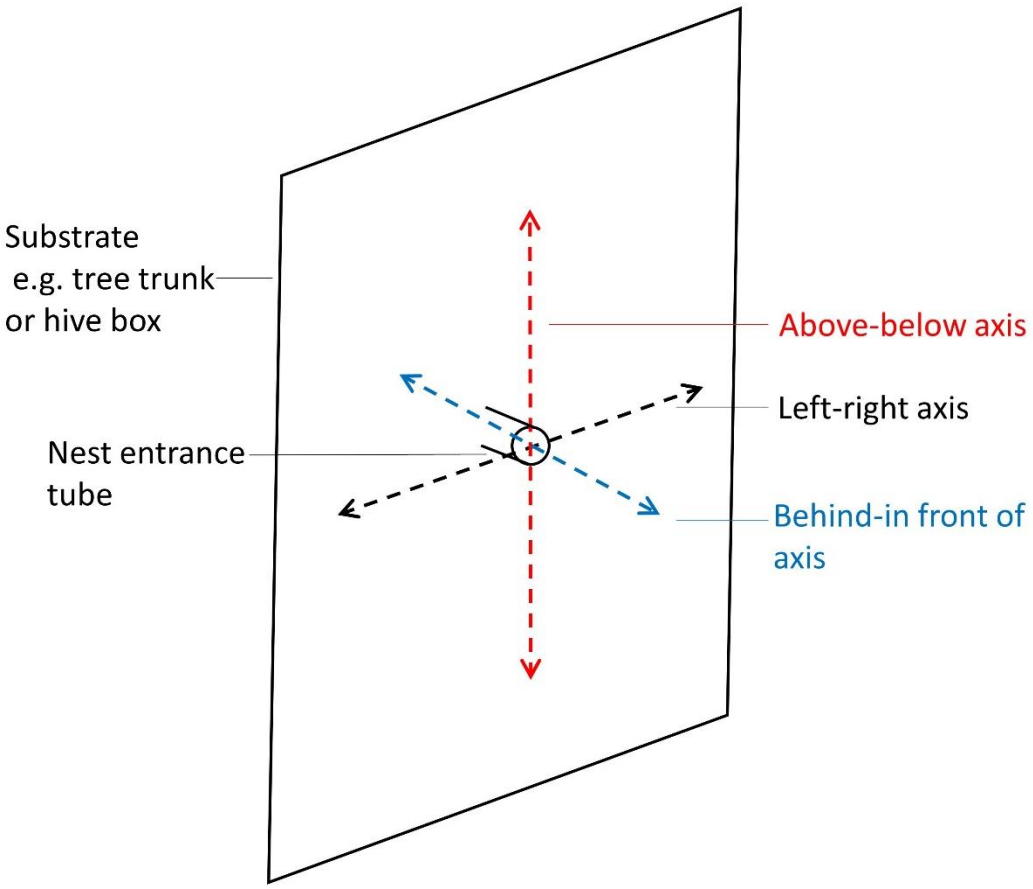

Figure 4.2: The three axes along which variation in the spatial position of *Tetragonisca angustula* hovering guards was measured relative to the nest entrance.

To determine the direction that hovering guards were facing, we used the same video approach as for determining three-dimensional position, and used the angle tool in Image J to measure the facing of hovering guards relative to the nest entrance. Watson's goodness of fit test was used to test if circular distributions differed from a uniform circular distribution (Lund & Agostinelli 2012). If circular distributions differed from uniform, then Hartigan's dip test was used to test for multimodality of the data, i.e. did guards tend to face in one direction or in more than one direction (Hartigan & Hartigan 1985, Maechler 2015). Finally, Watson's test for homogeneity was used to test for differences in circular distribution between different groups of bees, for example, those on different sides of the entrance (Lund & Agostinelli 2012).

Distribution along individual axes

After determining the three-dimensional position of hovering guards, our aim was then to investigate organisation along the axis or axes with the greatest variation. We made more extensive counts of hovering guards at the nest entrance of each colony, for guards in groups of 2, 3 and 4, because these were by far the most common (see Results). For each guard number (2, 3 or 4), the frequency with which guards were distributed along the axis on either side of the entrance, side A versus side B, was then compared with the expected random distribution. That is, if the probability of each individual being positioned on side A or side B was 0.5. Expected distributions were calculated using the binomial theorem with the formula $(p + q)^n$ where p is the probability of a bee being observed on the side A (0.5), q (= 1 - p) is the probability of a bee being observed on side B (0.5) and n is the total number of bees. For example, for two bees the formula $(p + q)^2$ can be expanded to $p^2 + 2pq + q^2$. This equates to probabilities of $0.5^2 = 0.25$ two bees on side A, $2 \times 0.5 \times 0.5 = 0.5$ one bee either side, and $0.5^2 = 0.25$ two bees on side B.

Vigilance of hovering guards

We investigated the effect of guard number and arrangement on vigilance. Most previous studies have measured vigilance by the degree of scanning, in which an individual raises its head and surveys its environment (Elgar 1989), or by the time taken to consume food items (Lima 1995). The small size of *T. angustula* (body length 4-5 mm) makes measuring head movements in the field impractical. Furthermore, guards do not forage, spend all of their time guarding, and are presumably always 'scanning'. However, guards often change orientation as they hover. We therefore quantified body rotation as a measure of individual vigilance (Ward et al. 2011). Because hovering guards rotate considerably more laterally than longitudinally (see Results) we used our earlier video recordings and still images from directly above the entrance to compare range of lateral rotation among guard groups of varying number (n = 33, 38, 24 and 24 guards for groups of 1, 2, 3 and 4 guards respectively). This range does not represent the total field of view of the bee, because the eyes are situated on the side of the head providing a wider view of the environment than human vision. Rather, the range represents the degree to which each bee moved and so increased its view of the

environment. To analyse these data, we ran a mixed-effect model fitting range as the response variable, guard number as a fixed effect (a factor with levels 1, 2, 3 and 4) and colony as a random effect, followed by *post hoc* multiple comparisons.

To quantify the collective vigilance of the guard group as a whole, we calculated the cumulative angle covered by each of the above groups (n = 33, 19, 8 and 6 for groups of 1, 2, 3 and 4 guards respectively). The angles of all individual guards within a group were summed minus any overlap in ranges. That is, if bee A and bee B both cover a range of 90° with no overlap in field of view, then the collective vigilance of the group is 90+90=180°. If bee A and bee B both cover a range of 90° but overlap in their field of view by 20°, then the collective vigilance of the group is 90+90-20=160°. We then ran a mixed-effect model fitting total angle covered as the response variable, guard number (a factor with levels 1, 2, 3 and 4) as a fixed effect and colony as a random effect, followed by *post hoc* multiple comparisons.

Finally, we investigated the difference in angle coverage between groups of two guards in arrangements of 1:1 (one on either side, n = 19 pairs) and 2:0 (both on the same side, n = 8 pairs). We calculated the cumulative angle covered by each group as before, then ran a mixed-effect models total angle covered as the response variable, guard ratio (1:1 versus 2:0) as a fixed effect and colony as a random effect.

Detection of a model predator

To investigate the effect of guard orientation on the ability of a colony to detect predators, we studied the simplest configurations of hovering guards; a single guard (n = 39) and two guards (n = 40), one of either side of the entrance. As well as being common (see Results), these simple configurations allowed us to address two questions: First, for a single guard, what is the probability of the guard detecting an intruder when it approaches from the guard's front versus behind? Second, for two guards and an intruder approaching perpendicular to the entrance such that it is directly in front of one guard and behind the other, which guard detects the intruder first?

While the compound eyes of *T. angustula* cover a similar proportion of the head to *Apis mellifera*, and therefore presumably have a wider view of their environment than does a human (Seidl & Kaiser 1981, Merry et al. 2006). However, like many bees and other insects the vision of *T. angustula* probably does not extend 360 degrees, and instead has an area of dead space at the centre of the posterior hemisphere of the head (Seidl & Kaiser 1981, Merry et al. 2006). That is, they have difficulty seeing directly behind them. Furthermore, the density of ommatidia is lower towards the posterior hemisphere of the eye such that resolution is lower (Land 1997, Land & Nilsson 2012), meaning objects approaching from the rear are more difficult to detect. Last, smaller bees have smaller eyes with lower resolution (Jander & Jander 2002), making the visual detection of small moving objects such as other bees very difficult until the object is near.

We simulated the attacks of *Lestrimelitta limao* robber bees using a dummy bee made of black modelling clay (10×3×3 mm), following van Zweden et al. (2011). The dummy was treated with citral (Sigma Aldrich, Stenheim, Germany) a major component of *L. limao* mandibular glands and known to elicit aggressive defensive responses in *T. angustula* (Wittman 1990, van Zweden et al. 2011). We suspended the dummy from a wooden pole via a thread (diameter 0.3 mm), and introduced it perpendicular to the colony entrance, directly in front of and/or behind the hovering guards, not head-on to the entrance as in van Zweden et al. (2011). To elicit an attack from *T. angustula* we began moving the dummy towards the colony entrance from an initial distance of 20 cm at a constant rate of 1 $cm.s^{-1}$ until an attack occurred. An attack was defined as a hovering guard flying directly towards and grasping the dummy, at which point the trial was terminated. If the dummy reached the entrance without receiving an attack from the bees then the trial was terminated. Each trial used a fresh dummy.

General statistical methods

All statistical analyses were conducted using R version 3.3.2 (R Core Team 2016), including the packages lme4 for mixed-effects models (Bates et al. 2015) and lsmeans for *post hoc* tests (Lenth 2016). P-values and test statistics are reported from model comparison between the minimal adequate model and null model, using the in-built ANOVA function of R.

4.4 Results

Three dimensional positioning and facing of hovering guards

Hovering guards showed variation in their distribution along all three axes. Relative to the nest entrance, the maximum, minimum and range of distributions in mm were 5.0, -19.7, 24.7 on the above–below axis (n=14), and -12.9, 41.3, 54.2 on the behind–in front axis (n=119) (Figure 4.3, 4.4). However, by far the greatest source of variation was along the left–right axis (-93.6, 116.9, 210.5, n=119), 388% and 852% more than the behind–in front and above–below axes respectively (Figure 4.4).

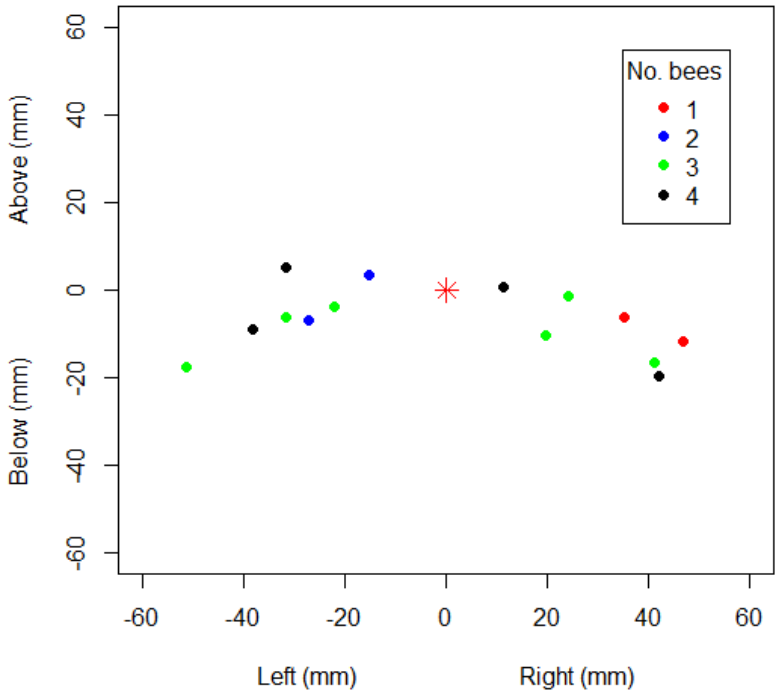

Figure 4.3: Spatial distribution of *Tetragonisca angustula* hovering guards (circles) relative to the nest entrance (red asterisk) in two dimensions, left─right of the entrance and above─below the entrance for groups of 1-4 bees.

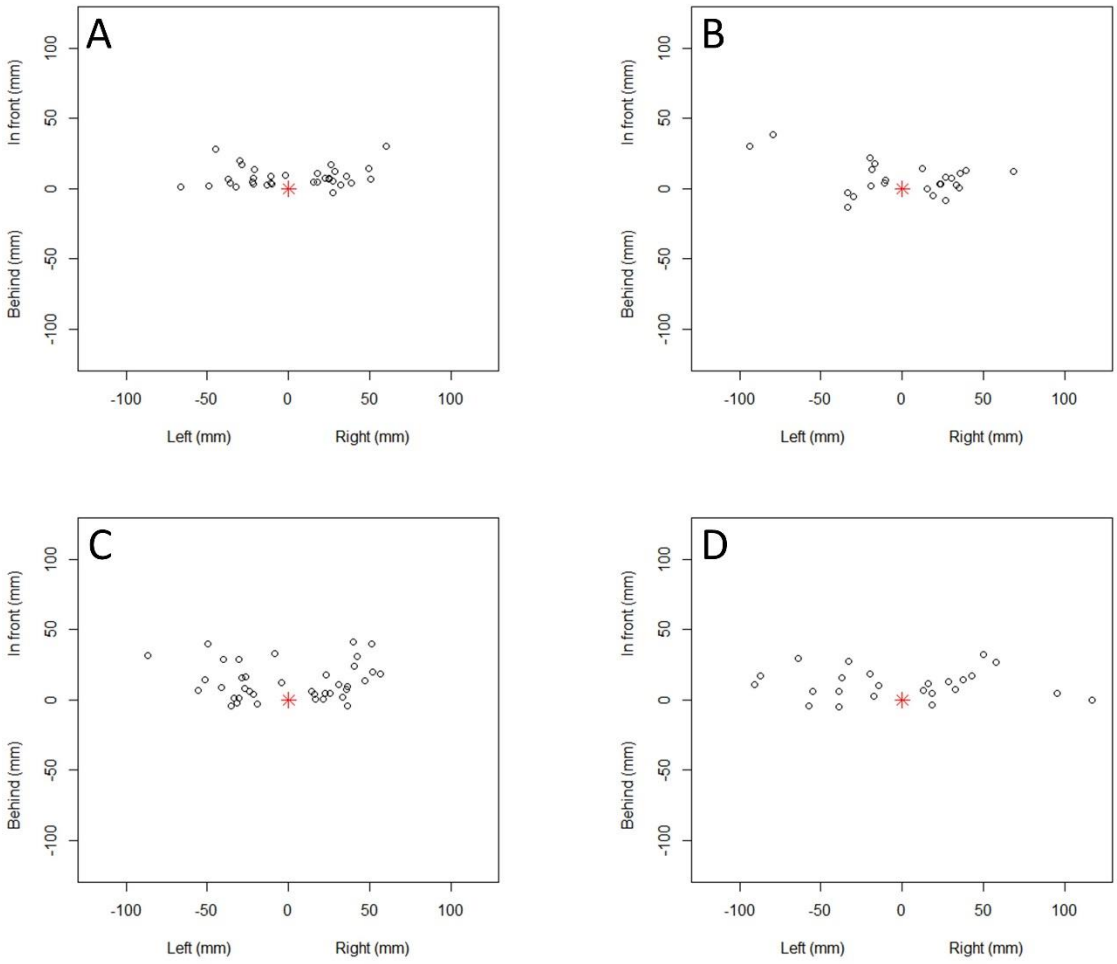

Figure 4.4: Spatial distribution of *Tetragonisca angustula* hovering guards (open circles) relative to the nest entrance (red asterisk) in two dimensions, left─right of the entrance and behind─in front of the entrance for **A** one bee, **B** two, **C** three and **D** four bees.

Significantly more hovering guards were positioned in front of the entrance than behind it (106 versus 13, chi-squared test, χ^2=72.68, DF=1, P<0.001), and significantly more were positioned below the entrance than above it (3 versus 11, chi-squared test, χ^2=4.57, DF=1, P=0.033). However, there was no difference in the number of hovering guards between the left and right of the entrance (59 versus 60, chi-squared test, χ^2=0.008, DF=1, P=0.927).

With regards to direction of facing, preliminary observations indicated that hovering guards were near horizontal, tilted slightly upwards towards the nest entrance, mean

angle from zero±SD = 87.8±3.1°, n=10. The small standard deviation suggested only minor longitudinal variation (i.e. little tilting of the head up and down) and this was confirmed by calculating the mean of the ranges of circular distributions, 3.7±2.9°, n=10. We observed far greater rotation in the lateral plane, pivoting side to side, mean range 29.7±16.0°, n=119, 802% more rotation than in the longitudinal plane (Figure 4.5). Therefore, we focussed on scanning behaviour in the lateral plane.

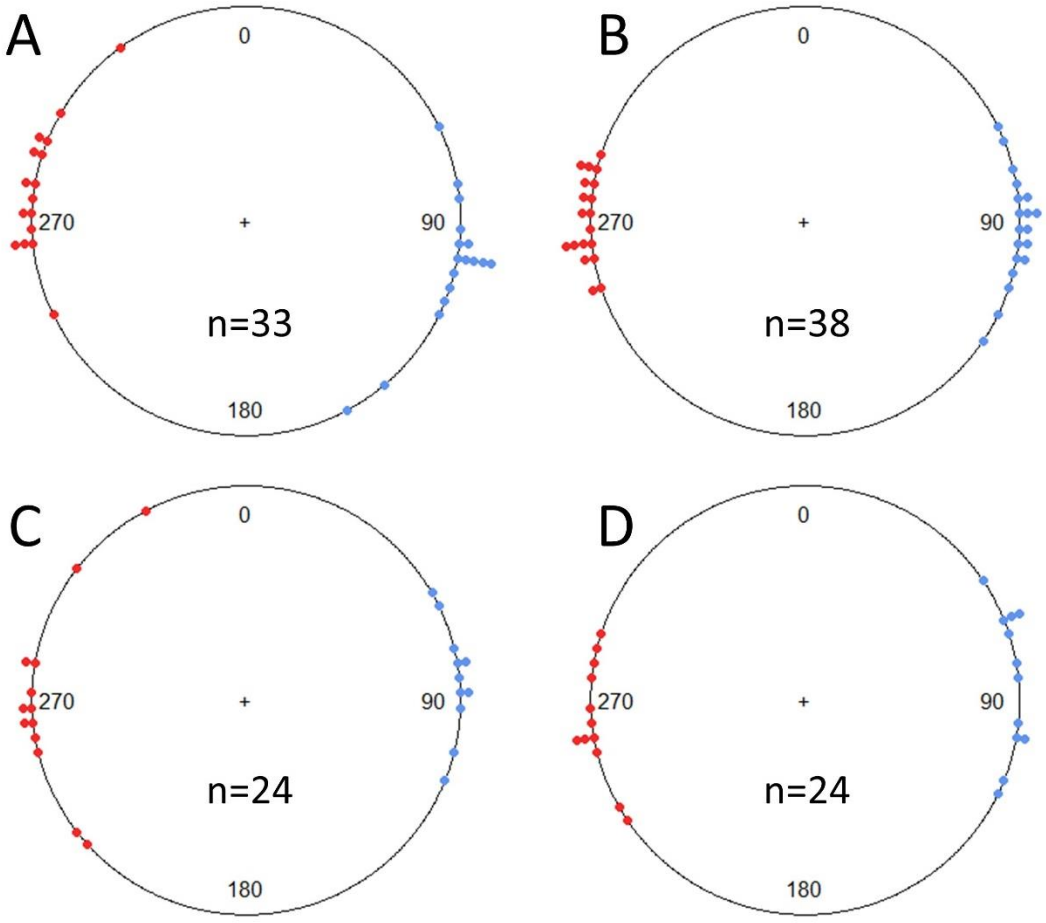

Figure 4.5: 360 degree facing of *Tetragonisca angustula* hovering guards at the nest entrance when viewed from above for arrangements of **A** one, **B** two, **C** three and **D** four bees. Red dots indicate bees on the right of the entrance (i.e. they tend to face left), blue dots indicate bees on the left of the entrance (i.e. they tend to face right).

Hovering guards were not uniformly distributed about 360 degrees (Watson's test for circular uniformity, U^2=1.140, P<0.01) and their angular distribution was not unimodal (Hartigan's dip test, D=0.155, P<0.001), i.e. the disribution was at least bimodal. Visual

inspection of the data (Figure 4.5) revealed that facing was aggregated in two groups. Those bees to the left of the entrance positioned their bodies such that they faced right, with a mean angle from zero±SD of 92.1±18.7°, n=59, while hovering guards on the right of the entrance faced left, 272.0±18.8, n=60 (Figure 4.5). Watson's two sample test of homogeneity confirmed that bees on the left and right of the entrance face in different directions (U^2=2.480, P<0.001). That is, hovering guards face inwards and those on the left and right of the entrance have a near diametrically bimodal circular distribution.

We can now give the location of a hovering guard, using the mean of its position along the three axes, combined with those of its facing. That is, a "typical" hovering guard is positioned 11.4±10.0 mm in front of the entrance, 8.4±5.8 mm below the entrance, and 34.3±20.3 mm on either the left or right of the entrance. It should be emphasised that these values are for data collected on colonies with ≤4 hovering guards, which is common. However, in colonies with a greater number of hovering guards, those guards are likely to be spread out over a greater perimeter (van Zweden et al. 2011). Guards then face inwards, perpendicular to the nest entrance, and slightly upwards, forming a corridor through which bees entering the nest fly.

Distribution along individual axes

In total, we made 287 observations of our 15 nests. The most common number of hovering guards present was one (23.7%), followed by two (23.0%), three (16.0%), zero (12.9%) and four (7.7%). In the remainder of observations there were ≥5 guards (16.7%). We found no overall bias for guards to be positioned on either the left or right of the entrance, 406 left versus 411 right (chi-squared test X^2 = 0.031, DF = 1, P=0.861). This validated our random model (see Methods), in which $p = q = 0.5$, and allowed us to combine inverse ratios. For example, in an arrangement of four guards, the counts left:right of 1:3 and 3:1 were pooled.

Hovering guards were significantly more likely to be distributed evenly on both sides of the entrance (left and right) than randomly. This was true for all arrangments for which our sample size was sufficient; two (X^2 = 29.333, DF = 1, P < 0.001, n = 66, Figure

4.6A), three (X^2 = 6.522, P = 0.011, DF =1, n = 46, Figure 4.6B) and four guards (X^2 = 15.303, DF = 2, P = 0.001, n = 22, Figure 4.6C). The sample size for arrangments of ≥5 guards was too small for analysis.

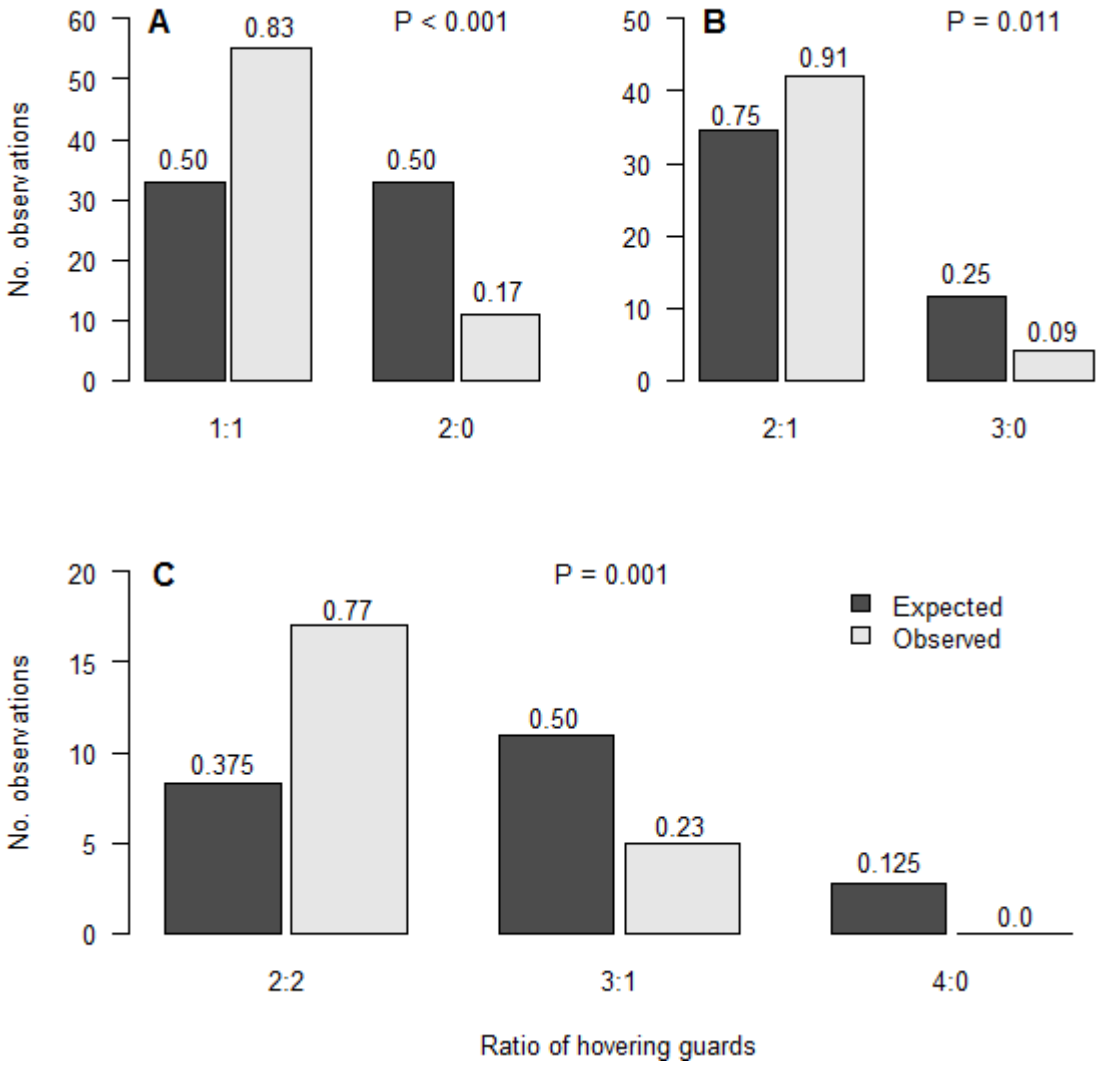

Figure 4.6: Observed and expected numbers of hovering guards of *Tetragonisca angustula* for arrangments of **A** two guards, **B** three guards and **C** four guards. Symmetrical arrangements are combined for example, 2:0 with 0:2. Numbers above bars indicate proportions, P-values indicate overall significant differences in observed versus expected numbers for each arrangement.

Vigilance of hovering guards

Guard number had a significant effect on the lateral scanning behaviour of guards (Figure 4.7A, mixed-effects model, X^2 = 11.513, DF = 3, P = 0.009). Lone guards

rotated laterally 42% more than guards in groups of two or more (38.1±15.8° compared to 26.7±13.3°). *Post hoc* multiple comparisons found significant differences in rotation between lone guards and those in groups of either two (P = 0.047) or three (P = 0.017). There was no significant difference in rotation between one and four guards (P = 0.074), and no significant differences among guard numbers greater than two (P > 0.05 in all cases).

Collective vigilance in hovering guards increased significantly from one to four guards (Figure 4.7B, mixed-effects model, $X^2 = 25.25$, DF = 3, P < 0.001). Guards in groups of four had a collective range of 83.0±29.1° compared with 38.1±15.8° for lone guards, more than double. This was largely due to having guards on both sides of the entrance. Finally, for groups of two guards, collective vigilance was over 50% greater when guards were present on opposite sides, 56.3±27.3°, versus the same side, 34.3±7.0° (Figure 4.7C, mixed effects model $X^2 = 4.487$, DF = 1, P = 0.034). That is, two guards arranged 1:1 have a greater view of the environment than when arranged 2:0.

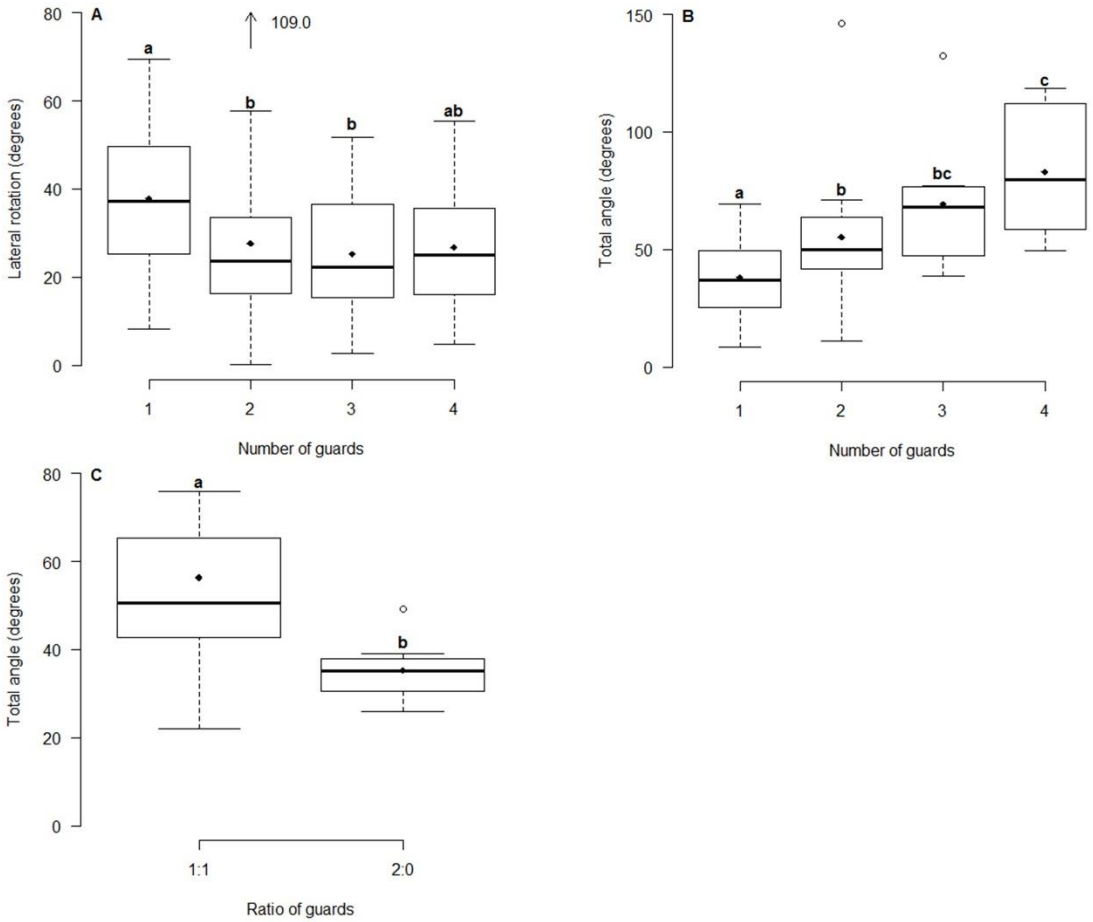

Figure 4.7: Vigilance in hovering guards of *Tetragonisca angustula* bees with varying guard number, measured as degree of scanning or lateral rotation for **A** individual guards, **B** cumulative angle covered by all guards not including any overlap and **C** cumulative angle covered by groups of two guards in 1:1 (opposite sides of the entrance) and 2:0 (the same side) ratios. Range calculated from the maximum minus minimum angle relative to the nest entrance from five snapshots of the position of a guard. Whiskers are 1.5× interquartile range. Diamonds indicate means, letters indicate significance following *post hoc* tests, circles and arrows indicate outliers.

Detection of a model predator

In contrast to van Zweden et al. (2011), *T. angustula* hovering guards did not always attack the dummy intruder, c. 50% in this study versus 100% in van Zweden et al. (2011). This may be because of differences in our methodology, as we purposefully used colonies with a small number of guards (1 or 2) and introduced the intruder at more difficult angles to detect, That is, from the side rather than head on.

Single hovering guards presented with a dummy intruder were three times more likely to detect and attack it before it reached the nest entrance when it approached from the front versus from behind the guard (Figure 4.8A). This difference was significant (mixed-effects model, $X^2 = 9.52$, DF = 1, P = 0.002, n = 58). When we presented the dummy to two guards, one on either side of the entrance, the dummy was twice as likely to be attacked by the guard facing the dummy, as opposed to the guard facing away (Figure 4.8B, chi-squared test, $X^2 = 4.333$, DF = 1, P = 0.037, n = 39). This result is especially striking, given that the guard facing the dummy was also the further from the dummy. Together, these results confirm that guards are better able to detect intruders that approach from the front rather than behind.

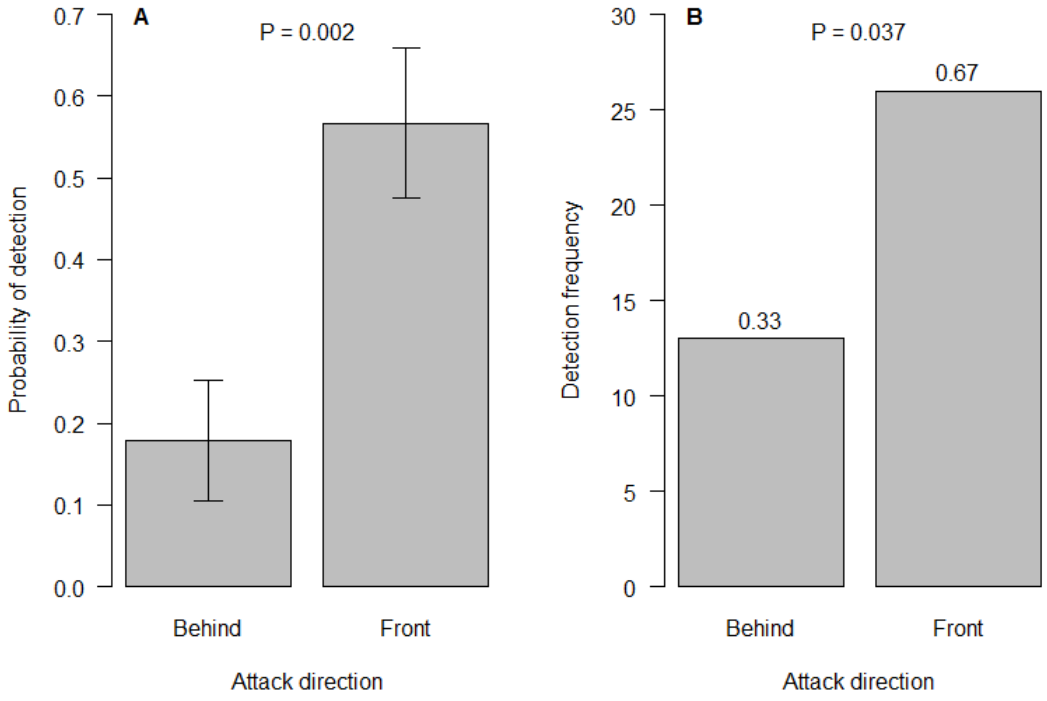

Figure 4.8: Ability of hovering guards of *Tetragonisca angustula* to detect predator models approaching from directions perpendicular to the colony entrance when the number of hovering guards is **A** one and **B** two, one on either side of the entrance. When one guard was present, a dummy predator was introduced either in front of or behind the guard, and the probability of the guard attacking the dummy was recorded. When two guards were present, the attacking bee was recorded as either the one facing (front) or not facing (behind) the dummy. P values indicate significance, numbers above bars indicate proportions.

4.5 Discussion

Hovering guards of *Tetragonisca angustula* coordinate themselves in a way that improves the collective vigilance of the group. Hovering guards formed a defensive corridor by distributing themselves either side of the nest entrance along the left-right axis, and by facing towards the entrance. Along the left-right axis, hovering guards were distributed more evenly, left versus right, than would be expected if each individual were positioned at random. This effect was significant in each of three situations; two, three and four guards, providing strong evidence for colony-level adaptive organisation. The effect was weaker when three guards were present. However, this was likely because the expected frequency of guards in the most even ratio (2:1) was 75%, meaning the maximum possible effect size in the direction of evenness was only 25% (Figure 4.6B) versus 50% (50% expected) when two guards were present (Figure 4.6A).

Collective vigilance increased as a result of both group size and the coordination. As hovering guard number increased, the total field of view covered by the group also increased (Figure 4.7B). This effect was greatest between one and two guards, as the second guard was typically on the opposite side to the first, which generally doubled the total field of view. Hovering guard groups in even arrangements had greater fields of view of the environment than those with skewed arrangements (Figure 4.7C). For two guards in a 1:1 ratio, and so facing in opposite directions, the second guard almost doubled the field of view of the group. However, when both guards faced in the same direction in a 2:0 ratio, it created considerable overlap between them. In a new example of the group size effect, individual vigilance decreased as hovering guard number increased (Figure 4.7A). This may be an adaptive response resulting from an awareness that other hovering guards were present. Alternatively, the increased level of rotation in small groups may be because every guard has to inspect incoming bees, whereas in large groups, some inspect while some remain in position and so rotate less.

Hovering guards seldom face outwards from the nest entrance, but this should not greatly limit the group's field of view of the environment, because the compound eyes of *T. angustula* extend to the side of the head and cover a wide area (Seidl & Kaiser 1981, Grüter et al. 2012). This allows the bee to see outwards even when its body is

perpendicular to the nest entrance. Coupled with the generally poor visual acuity of the insect compound eye (Mallock 1894, Kirschfield 1976, Snyder 1977, Land 1997), this suggests that the addition of guards facing outwards would not greatly increase predator detection. The positioning of hovering guards to face a flight corridor has the additional function of increasing the ability of guards to intercept intruders flying towards the entrance (Wittman 1985).

Guards facing in the direction of attack were better able to detect intruders, as shown in our two complementary bioassays. Lone guards were three times as likely to detect a dummy robber bee when it approached from the front, rather than the rear. When there were two hovering guards, the guard facing the intruder was twice as likely to initiate an attack as the guard facing away. This second result is all the more powerful because the guard facing the model predator was always the further from it of the two. While individual bees have blind spots in their visual field (Seidl & Kaiser 1981), the coordination of vigilance would seemingly reduce eliminate blind spots from the collective visual field of the group. The diffuse nature of larger guard groups may lead to the breakdown of this rule, because an intruder will have to bypass several guards facing away before it encounters a guard facing towards it. The direct defensive benefits of coordinated over non-coordinated vigilance is a topic for further study. In particular, it would be valuable to investigate whether coordinated vigilance in *T. angustula* increases the ability of a colony defend against the robber bee *Lestrimelitta limao*, which is probably the most important enemy of *T. angustula* (Segers et al. 2016, Grüter et al. 2017a). Furthermore, for a given level of collective vigilance, does coordination allow the colony to reduce its investment in guards?

The benefits of coordinated vigilance relative to the more established role of group size remain unknown. While we studied groups of 1-4 hovering guards, the number may exceed 15 (van Zweden et al. 2011). We predict that as group size increases, the importance of coordination relative to group size will diminish for two reasons. First, because coordination will become more difficult, analogous to the costs of monitoring other group members proposed by Ward (1985). Second, with many guards even a random configuration would likely cover all directions. Furthermore, as guard number

increases we expect collective vigilance (Figure 4.7B) to plateau as it approaches the limit of 360 degrees. However, higher guard number could still increase collective vigilance through the occupation of a greater area (van Zweden et al. 2011). There would also be defensive benefits unrelated to vigilance, in particular the ability to fight, harass or confuse predators should continue to increase with group size (Shields 1984, Landeau & Terborgh 1986, Krakauer 1995, Shackleton et al. 2015).

How do hovering guards achieve an even left–right distribution? We hypothesise that the pattern is self-organised, that is, individual guards react to their local environment and follow simple rules to produce the global pattern of being evenly distributed. Self-organisation is a common mechanism in insect societies, being used in the organisation of foraging, nest building and nest defence (Bonabeau et al. 1997, Millor et al. 1999, Boomsma & Franks 2006). Furthermore, there is evidence that self-organisation works in conjunction with group size to produce greater collective vigilance in fish shoals (Ward et al. 2011). The pattern in *T. angustula* could arise through individual guards applying two simple rules based on their local environment and experience: First, if a guard detects another guard on the same side of the entrance as itself, then its propensity to switch sides increases. Second, if after switching a guard detects another guard on the same side as itself, it remains for some time before moving, in order to prevent continuous switching. Alternatively, a guard may react to the absence of guards on its side, or the guard state on the opposite side to itself. There is some evidence that bees can count, at least up to four (Chittka & Geiger 1995, Dacke & Srinivasan 2008), which might also be used in distributing hovering guards into an even ratio.

An alternative hypothesis is that hovering guards are either left or right specialists, and that colonies have an equal number of specialists for the left and the right. Specialists could arise epigenetically or through experience. Epigenetic determination seems less likely, because it is less robust to the loss of guards. For example, if a colony loses all of its specialists on one side then replacing them will take longer. Specialisation through experience could arise through a naïve guard beginning its bout on one side at random, and then returning to that side in each subsequent bout. There could also be a combination of self-organisation and specialisation. Consider a situation with a lone

guard, and a second, naïve guard beginning its role for the first time. Under self-organisation, the naïve guard would join on the opposite side to the first guard, but then through experience may learn to stay on that side. For the same reason that experience seems more likely than epigenetic determination, self-organisation seems a more likely mechanism than specialists, because self-organisation would be more robust to change.

These two competing hypotheses for the organisation of hovering guards could be teased apart by marking bees individually and first observing the movements of guards over an extended period. Does a lone guard stay in its position, or switch to the other side of the nest, and does it return to the same side with each new bout of guarding? Do guards in a pair always appear in the same position relative to each other, or do they switch? Subsequent removal experiments could then be used to manipulate the left:right ratio of hovering guards and observe the response of the remaining guards. Do hovering guards return to the same side of the nest entrance, or do they organise themselves back into an even ratio?

In contrast to individuals in an ungulate herd, bird flock or fish shoal, social insect guards should always be vigilant. Because worker fitness is tied more closely to the colony than personal safety, guard groups should be free from the limitations of the selfish herd (Hamilton 1971), which may prevent the emergence of collective vigilance arising from the cooperation of unrelated individuals. In selfish herding unrelated individuals should strive for the safe positions and not regard the interests of their neighbours (Hamilton 1971, Morrell et al. 2011). The study of organised patterns in animals with high intra-group relatedness (for example, Santema & Clutton-Brock 2013) may reveal new rules governing vigilance and the benefits of group living in general.

Chapter 5 – Final discussion and future directions

The results in this thesis show some of the extreme and extraordinary defensive measures employed by stingless bees to protect their colonies and workforce, from crashing head first into a brick wall to suicidal biting. Because of the range of threats stingless bee nests are subject to, defensive responses must also be varied and appropriate.

5.1 Context of defence

One can move within centimetres of bees on flowers and they will continue to go about their business of foraging. However, if one approaches a group of bees at a nest then an attack is probable. This illustrates the importance of context for an animal in deciding what a threat is, and whether it is worth a response. Similarly, the type of threat faced by the colony is likely to mediate its defensive response. Threats take a variety of forms, and each will attack a colony in its own way and has the potential to inflict a different amount of damage. The bioassays used in Chapters 2 and 4 can be used to compare defensive response under two contrasting contexts, as they used different predator models to elicit defensive responses from colonies. Chapter 2 used a 10 × 10 cm black flag more closely resembling a small mammal or bird, while Chapter 4 used and a 1 × 0.3 × 0.3 cm clay model of a bee treated with the odour of a robber bee.

We can compare the response *Tetragonisca angustula* as it was studied in both chapters. *Tetragonisca angustula* attacked both the flag (47.5%) and clay bee (50%) at a similar rate. However, the flag was presented for 60 seconds versus only 20 for the clay bee, the median latency of attack for the black flag was 20 seconds, and only 55% of attacks occurred before 20 seconds. That is, over a period of 20 seconds, *T. angustula* attacked the flag only 26% of the time compared to 50% for the clay bee, almost half as often. Furthermore, the clay bee was purposefully introduced at an acute angle to the nest entrance, to make it difficult to detect. When van Zweden et al. (2011) performed a similar assay, introducing a clay bee to the front of the nest entrance, the attack rate was 100%. This suggests that *T. angustula* are more aggressive towards intruders resembling robber bees, than those resembling vertebrates. It is therefore possible our comparisons of aggression and self-sacrificial behaviour in Chapter 2, would have yielded different

results using a different type of model predator. This does not invalidate the results of Chapter 2, other than to recognise that some species may differ in their aggression under a different context. Future studies of defence should therefore, consider the context when measuring the defensive response.

Partamona helleri aggressively attacked the flag in Chapter 2, but when we attached dead jumping spiders to *Partamona helleri* nests in Chapter 3, the bees did not try to attack or remove them. Because spiders do not attack the colony but rather the individual workers, it may not be worth attacking the spiders if the benefits of doing so are not greater than leaving them alone. For example, workers may also be lost in attempting to fight and remove the spiders. While the detrimental effects of individual level predation on the colony may accumulate, they are considerably less than the potential damage from a large-scale attack on the colony.

The acceleratory and crash landing behaviour of *Partamona helleri* poses an interesting question on the trade-off between individual and colony safety. A narrow entrance requires greater accuracy and therefore lower speed to enter, which presumably leaves the bee more vulnerable to ambush predation. However, a narrow entrance is also more defensible against threats that target the colony rather than the individual (Couvillon et al. 2008a). A broader comparative study of nest entrance sizes, landing speed and behaviour, and predation rates (which is, admittedly, difficult to observe) would shed light on this issue. It is probable that colony safety is prioritised over individual safety, because even though attacks on the colony are probably rarer than individual level predation, the potential consequences are far more severe. Further questions may be asked about the amount of predation at the entrance suffered by *P. helleri* compared to other species without acceleratory behaviour, and how species without the acceleratory behaviour otherwise respond to ambush predation.

5.2 Correlates of aggression and self-sacrificial behaviour

Biting is a common defensive behaviour across the animal kingdom, even in other Hymenoptera that possess a sting. It is therefore possible that suicidal biting is more widespread than previously thought. The prerequisites for self-sacrificial biting would

appear to exist in many other social insect species. For example, leafcutter ants (Attini) have workers with powerful mandibles, and large enough colonies that the loss of a few workers would not be significant.

Questions still remain however, about the ultimate and proximate causes behind self-sacrificial behaviour. At an individual level, the behavioural switch between non-suicidal defence (where a bee bites then releases an intruder) and suicidal defence (where a bee bites and refuses to release an intruder) is poorly understood, but is of general biological interest. Existing studies have focussed on identifying the genetic loci and neurological pathways involved in the stinging response of honey bees (Hunt et al. 1998, Hunt 2007). Stingless bees could perhaps compliment those studies on honey bees, because the nature of their self-sacrifice differs. Honey bees have a binary choice: Stinging causes damage to the intruder but guarantees self-sacrifice, but choosing not to sting gives the bee no alternatives to damage the intruder i.e. there is no option to damage the intruder and not commit self-sacrifice. Honey bees can bite intruders but their mandibles are weak and not painful at all to a human. However, a biting defence in stingless bees may be viewed as a continuous variable of severity, along which there is a threshold for self-sacrifice.

The ultimate causes of self-sacrifice and aggression are likely linked to the trade-off between risk to the colony from the predator and maintaining the colony workforce. Colony size is a likely correlate of aggression and self-sacrificial behaviour, because workers from a large colony have lower residual value than those from a small colony. Individual defensive ability is also likely important, because if a worker is physically incapable of incapacitating or otherwise hindering an attacker then there is little value in being aggressive. Species that nest in exposed locations may be more aggressive, because their nest is more vulnerable to attack and launching a defensive response when an intruder is first sited may prevent it reaching and damaging the nest. Nests located in sheltered locations such as a tree cavity have a natural layer of defence and early aggression against a potential intruder is perhaps less necessary. Chapter 2 is suggestive of large colonies being more aggressive, but the largest and most aggressive colonies all belong to the same genus, *Trigona*. A wider dataset encompassing more species and

analysed using phylogenetic contrasts could reveal the correlates of aggressive and self-sacrificial behaviour. In this regard, within species comparisons would also be of great value.

5.3 Costs and benefits of aggression in defence

In Chapter 2, I found that stingless bee species exhibited a wide range of aggressive responses to our stimulus, a black flag. Some species never attacked while others attacked 100% of the time, even though the flag itself was not a genuine threat. Similarly, *Trigona* spp. often attack humans in the vicinity of their nest, even if that human is non-threatening. Such aggression against innocuous targets may constitute 'false alarms' (Beauchamp 2010, Stephens et al. 2017). False alarms can be costly because defenders may be lost attacking a target that is not a real threat. While there is little doubt that aggression through mass attacks are a potent deterrent for any would be predators, the behaviour may be maladaptive in an environment where the actual threat of predation is low. In Chapter 2, I made the argument that the death of 20 workers from a 10,000 strong *Trigona* colony (0.2% of the workforce) represented an acceptable loss for the successful repulsion of an attack. However, these losses will become significant if they are repeated over the course of the day due to false alarms.

The decision of whether to attack draws parallels with the acceptance threshold model of nestmate recognition (Reeve 1989). Do colonies attack any foreign animal they detect, losing a few workers each time? Alternatively, do colonies adopt a more permissive approach, by not attacking every potential threat but risking a genuine threat approaching and damaging the colony? The ability of a colony to respond accurately to potential threats should therefore be under strong selective pressure. It would be interesting to test the relative costs and benefits of aggressive versus non-aggressive defence across environments with varying levels of predation. Of course, quantifying predation is difficult because predation events are rarely observed under natural conditions. However, the predation pressure experienced by study colonies can be manipulated by the translocation of hives to areas of known natural enemy populations (Segers et al. 2016). One prediction is that non-aggressive colonies would perform better in environments where the proportion of false alarms compared to genuine attacks

is high, because few workers are lost needlessly leaving more available for other duties. In environments with greater levels of predation, non-aggressive colonies may suffer, as they are less able to defend their nests.

5.4 Advances in the study of vigilance behaviour

A large proportion of the work on vigilance concerns vertebrates, particularly birds and to a lesser extent mammals. However, nearly all animals suffer the threat of predation and the early detection of predators increases chances of survival. I have shown in Chapter 4 how a behaviour that is rarely observed or unlikely to evolve, coordinated vigilance, has great relevance for a eusocial insect in defence of its nest.

Coordinated vigilance may be rare because group members are selfish or that it is costly to monitor the vigilance of other group members (Hamilton 1971, Beauchamp 2017). However, coordinated vigilance behaviours may evolve in species where intragroup relatedness is high, such as social insects. It would be interesting to compare vigilance among groups that varied in their intragroup relatedness. The stingless bees are perhaps not well suited to this question, because colonies are nearly always headed by a single queen that is nearly always singly mated, so there is low variability among colonies in intracolony relatedness (however, there may be some variation in intracolony relatedness owing to the proportion of worker laid eggs (Wenseleers & Ratnieks 2004)). Furthermore, workers have already sacrificed reproduction, meaning that guards would always be expected to be vigilant.

These questions may be better addressed by using study systems in mammals or birds with a more dynamic social structure. For example, in well-studied groups of meerkats where each animal is individually known and recognisable (Jordan et al. 2009), the relatedness of one individual to another could be calculated or otherwise obtained by sampling DNA, and the levels of vigilance compared both among members of the same group and among different groups. A study such as this would require that individuals have some ability to recognise their kin. Meerkat and other mongoose societies are also fluid, with dispersal and forced evictions commonplace, meaning that intragroup relatedness naturally varies among groups (Stephens et al. 2005, Thompson et al. 2017).

One could also manipulate the group composition of captive populations to compare vigilance among between related and completely unrelated individuals.

5.5 Organisation beyond hovering guards

Chapter 4 showed how the spatial organisation of defenders could lead to a more effective defence of the nest. I used the hovering guards of *T. angustula* as a model system, but such spatial organisation may be far more widespread among social insects. For example, guards of many stingless bee species sit upon the lip of the nest entrance and appear to have a somewhat uniform 360 degree distribution, although there is perhaps some bias towards the lower lip (for example, Figure 1.2C, 3.1C, 3.1E). This would have the benefit of being able to intercept bees that arrive from any direction, although forager traffic is likely to be concentrated on the lower lip. The spatial distribution of guards about the nest entrance could easily be tested for many species that build approximately circular nest entrances. Further questions may then be asked about the defensive strategy guards adopt when the number of guards is limited, such that the whole circumference of the entrance cannot be occupied. Similar experiments to those suggested in the discussion of Chapter 4, involving the individual marking and removal of guards, could be performed to reveal the mechanisms behind the spatial organisation of defence in social insects and other animals.

A broader question concerns quantifying the value of organised defence. In the case of vigilance in *T. angustula*, the organisation of guards helps to detect predators (Chapter 3), but so can simply increasing guard number (van Zweden et al. 2011). The question may be viewed in terms of the colony's defensive investment, because every additional guard is one less forager. That is, for a given level of defence required by a colony, how many more guards are required for a non-organised group than an organised group? Alongside the ability to detect threats, we must also consider the value additional guards add to the ability of a colony to fight threats once detected. Vigilance is likely important during the initial stages of a raid when it is possible to disable a scout, but once underway a raid may conform to Lanchester's laws of combat, where numerical superiority or individual fighting ability are of greater importance (Lanchester 1956, McGlynn 2000, Adams & Mesterton-Gibbons 2003).

5.6 Soldier specialisations

Soldiers, by definition, are morphologically distinct from other workers such as foragers and nurses, being specialised for defence. This can be seen in the disproportionally large heads of leafcutter ant majors, and the more moderate differences in soldiers of *T. angustula* (Grüter et al. 2012). Some differences between soldiers and other workers may be more subtle, however. For example, *T. angustula* soldiers have more sensilla on their antennae, which increases their ability to detect the chemical cues of intruders (Grüter et al. 2017b). *Tetrangustula angustula* soldiers also have larger eyes (Grüter et al. 2012), which presumably gives them better visual resolution (Jander & Jander 2002). Workers of many bees and other flower visitors have a band of increased visual acuity towards the front and around the equator of the eye, which is thought to increase their ability to see flowers (Land 1997, Land & Nilsson 2012). Because soldiers do not forage on flowers, but rather use their eyes to detect incoming nestmates and intruders, we may expect differences in the density of ommatidia across the eye between soldiers and other workers. In particular, because hovering guards of *T. angustula* face perpendicular to the nest entrance, it would be interesting to investigate whether they possess an acute zone more laterally than forager bees.

5.7 Organisation from the attacker's perspective

There is a growing body of literature on nest defence, for which the stingless bees are a rich source for discoveries. However, we must also consider the interaction from the perspective of the predator or parasite. A recent review of the literature suggests that robber bees select victim colonies that are smaller, have higher quality honey, lower defensive abilities, and are chemically similar to themselves (Grüter et al. 2016). However, the initial stages of an attack are very rarely observed (Christoph Grüter, Denise Alves pers comm), and so many questions remain about how attacks are organised, and the trade-offs between selecting a rewarding target and the risks to the attackers (loss of their own workers).

For example, Chapter 4 showed the benefits of having guards on both sides of the entrance in *T. angustula*. However, without observing the initial stages of an attack, it is

difficult to discern the adaptive significance of this organisation. What strategy does the first *L. limao* scout employ when it approaches the victim nest? Does it rely on its physical advantages to overpower the defenders or does it attempt to gain some positional advantage? Furthermore, because *L. limao* attacks numerous victim species, how does its attack strategy vary with the wide variety of defences it must face? Finally, how does *L. limao* modulate its recruitment during a raid? Honey bees can incorporate multiple variables of risk and reward to finely tune the recruitment of nestmates to flowers via the waggle dance (von Frisch 1967, Seeley 1995, Abbott & Dukas 2009). Can *L. limao* modulate its recruitment in response to reward and the strength of host defences in a similar way?

While Chapter 4 and much of the literature on robbing behaviour considers the interactions of colonies with the obligate robber *Lestrimelitta limao*, a great deal of robbing also occurs between non-obligate robbers. The consequences of the raid are similar but what is innocuous at one time may be a significant threat at another. Raids are likely to occur between colonies of the same, or closely related species, because the similarity of chemical recognition cues makes it easier to remain undetected by the guards of the victim colony. It would be interesting to compare the strategy and rate of success of facultative robbers with that of *L. limao*.

5.8 Final thoughts

The interactions between predator and prey, and between parasite and host, form the basis for countless questions in a variety of biological fields including behavioural ecology, evolutionary biology, chemical ecology and mathematical modelling. This thesis has addressed questions in behavioural ecology, highlights how the stingless bees, a relatively understudied group, can be used to answer these questions, and presents several new avenues of research.

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